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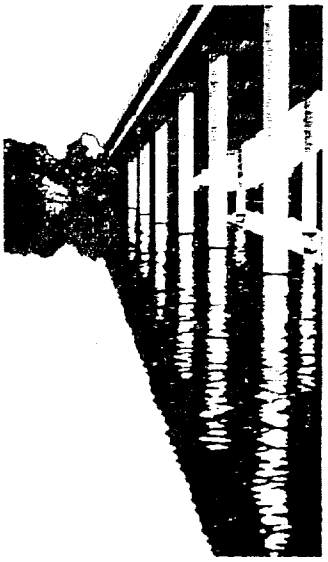
MICROBIOLOGICAL CONTROL OF EURASIAN WATERMILFOIL

FINAL REPORT

by

Haim B. Gunner, Yuthana Limpa-amara, Beryl S. Bouchard
Philip J. Weilerstein, Mark E. Taylor

Department of Environmental Health Science
University of Massachusetts
Amherst, Massachusetts 01003



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An attempt was made to develop an ecosystems approach for the control of the nuisance aquatic plant Eurasian watermilfoil, *Myriophyllum spicatum* L. To this end, a cellulolytic fungus, *Mycoleptodiscus terrestris* (Gerdemann) Ostazeski (*M. t.*), and a pectinolytic bacterium, *Bacillus* sp. strain P8 (BSP8), were isolated from the microbial populations naturally resident in the phyllosphere of this plant. These organisms grew compatibly with each other, were able to compete successfully with other microflora on the plant surface, and were able to resist the inhibitory action of phenolic compounds produced by the plants.

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Application of these organisms to the plant, after growth in appropriate media, resulted in plant decline and eventual death. The process of decline included hormonelike stress effects on the plant induced by BSP8 (internodal elongation), an increase in strongly pectinolytic microbial populations associated with the plant, and penetration into plant tissue by the fungal mycelium.

These results were confirmed in a sequence of experiments of increasing volume conducted in jar, pool, and ultimately, a lake setting. The most rapid decline of the plant, subsequent to inoculation, was observed in jars in the laboratory where a high concentration of inoculum and isolation from environmental effects could be maintained. In the pool setting, though the application of *M. t.* and BSP8 together induced the most significant decline, limits in pool depth and light penetration may also have influenced plant decomposition. Field applications, however, ultimately resulted in the virtual elimination of *M. spicatum* from a treated plot within 10 weeks.

Total numbers of microorganisms isolated from *M. spicatum* tissues were generally two to four orders of magnitude higher than numbers obtained from the adjacent water profile, reflecting the substrate commitment of the microorganisms to the plant. The numbers of organisms were greater on treated than untreated tissues, and parallel increases were observed in the water profile adjacent to treated plants. These results were confirmed by observation under the scanning electron microscope.

Specificity trials of *M. t.* infectivity revealed it to be very weakly pathogenic to several aquatic species and terrestrial plants, and thereby without potential significant impact outside the area of designated application.

PREFACE

The research reported herein was sponsored by the Headquarters, US Army Corps of Engineers (HQUSACE), through the Aquatic Plant Control Research Program (APCRP), Work Unit 32202, and was conducted under Contract No. DACW39-80-C-0029. Technical Monitor for the HQUSACE was Mr. James W. Wolcott. The APCRP is managed by the US Army Engineer Waterways Experiment Station (WES) under the Environmental Resources Research and Assistance Programs, Mr. J. Lewis Decell, Manager. The study was conducted under the general supervision of Mr. Edwin A. Theriot, Chief, Aquatic Habitat Group; Dr. C. J. Kirby, Chief, Environmental Resources Division; and Dr. John Harrison, Chief, Environmental Laboratory, WES.

The report was prepared by Dr. Haim B. Gunner and Messrs. Yuthana Limpa-amara, Beryl S. Bouchard, Philip J. Weilerstein, and Mark E. Taylor of the Department of Environmental Health Science, University of Massachusetts, Amherst, MA. Dr. Gunner was Principal Investigator. The report was edited by Ms. Jessica S. Ruff of the WES Information Technology Laboratory.

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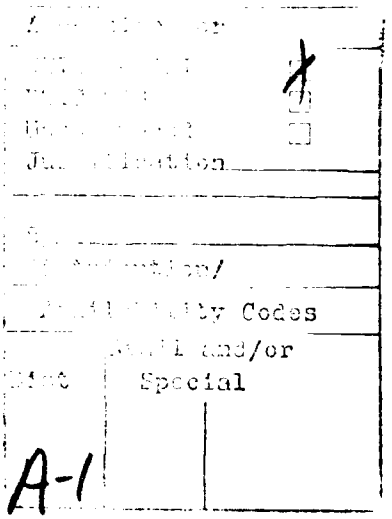

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MICROBIOLOGICAL CONTROL OF EURASIAN WATERMILFOIL

FINAL REPORT

PART I: INTRODUCTION

Background and Purpose

1. The severity of aquatic plant infestations has become a problem of growing national importance. Nuisance plants, such as *Myriophyllum spicatum*, the Eurasian watermilfoil, which two decades ago had already succeeded in covering over 25,000 acres (101 million m^2) in eight Tennessee Valley Authority reservoirs (Smith, Hall, and Stanley 1967), have now emerged as the significant aquatic plant nationwide. As a result, the development of appropriate control procedures has become a matter of genuine urgency (Hayslip and Zettler 1973; Aiken, Newroth, and Wile 1979).

2. Initially, chemical herbicides such as 2,4-D were reported to be an effective means of management even when used at low concentrations (Killgore 1984; Aiken, Newroth, and Wile 1979). However, mounting evidence of their toxicity and the danger they pose to other living systems has resulted in increasing restrictions on their use, particularly in sensitive water bodies (Amundsen and Brenkert 1978).

3. Mechanical control methods such as winter drawdown, bottom coverings, bottom tillage, and weed harvesting are also used, but application limits and economic considerations (Newroth 1985) restrict their use to specific situations (Bates, Burns, and Webb 1985; Newroth 1985).

4. A third approach to aquatic plant control is the application of plant pathogenic microorganisms (Charudattan 1975). In one instance, the fungus *Cercospora rodmanii* increased disease intensities in waterhyacinth after application as inoculum when compared with the naturally occurring endemic infestation. Two other pathogenic fungi, *Fusarium sporotrichoides* and *Acremonium curvulum*, were also shown to be potent biological agents in controlling Eurasian watermilfoil (Andrews and Hecht 1981; Andrews, Hecht, and Bashirian 1982; Patlak 1982).

5. This work has taken an ecosystems approach to the development of microbial control agents. The emphasis is on organisms naturally present in

the plant environment (Blotnick, Rho, and Gunner 1980). The contention here is that a search among plant-associated microflora can identify organisms with enzyme systems which, after appropriate growth procedures and inoculation back onto the plant, are capable of bringing about the decline and death of Eurasian watermilfoil.

6. The purposes of this investigation were to (a) isolate the microflora (cellulolytic and pectinolytic) associated with Eurasian watermilfoil and (b) characterize the parameters of their affiliation with this plant which, when appropriately enhanced, could be employed to constrain its growth and survival.

Literature Review

7. The presence of a phyllosphere effect, that is to say, the establishment of selected microfloral populations due to excretions from the host plant, is a well-established terrestrial phenomenon. In the terrestrial environment, where the focus has been on the impact of the zone of influence exercised by the root system or rhizosphere, the relationship is best described as protocooperation where both members benefit from nonobligatory coexistence. Beneficial effects of the rhizosphere microflora on plant growth may be summarized as (a) increased nutrient availability in the root zone (Estermann and McLaren 1961, Nicholas 1965, Rovira and Davey 1974), (b) positive effects on nutrient absorption rates (Barber and Frankenburg 1971), (c) production of plant growth stimulators (Katznelson and Bose 1959; Barea, Mavarro, and Montoya 1976; Alexander 1977), and (d) increased resistance to soil-borne plant pathogens (Harris and Sommers 1968, Alexander 1977).

8. Detrimental effects have also been shown to occur. These include the immobilization of limiting nutrients and the production of substances toxic to plant metabolism (Alexander 1977). More recently, there has been growing interest in the rhizosphere of aquatic plants. Coler and Gunner (1969) found higher populations of bacteria and concentrations of amino acids surrounding the roots of free-floating duckweed. Mahmoud and Ibrahim (1970) found a positive rhizosphere effect with nitrifying bacteria, i.e., an increase in numbers, that increased with the age of submersed rice plants, and a negative rhizosphere effect, or decrease in numbers, with denitrifying bacteria. Many authors have reported the presence of nitrogen-fixing bacteria in the

rhizosphere of aquatic plants. Patriquin and Knowles (1972) and Bristow (1974) found this to be an area of higher populations and enhanced activity for nitrogen-fixing bacteria. The aquatic rhizosphere is thus becoming an important area of research with respect to the growth of submergent aquatic plants, as well as food crops. Blotnick, Rho, and Gunner (1980) investigated the rhizosphere microflora of *Myriophyllum heterophyllum* and found a significantly denser bacterial population in the extensive rooting systems of this plant than in surrounding sediments, presumably prompted by greater access to organic nutrients in the sediments due to root exudates and sloughed off root materials.

9. As may be anticipated, nutrient availability is an important factor in determining the types and numbers of bacteria in aquatic systems, as elsewhere (White et al. 1979, Rheinheimer 1985). In clean lakes, the highest populations of bacteria are present at the time of the greatest production of nutrients by phytoplankton. This occurs in spring (Rheinheimer 1985), late summer (Niewolak 1974), early autumn (Jones 1973, Rheinheimer 1985), or early winter (Rheinheimer 1985). It follows, therefore, that the highest number of bacteria is detected in eutrophic lakes and reservoirs, while the lowest is found in oligotrophic lakes (Rheinheimer 1985). With respect to the distribution of microbial populations in aquatic systems, it has also been observed that, in stratified lakes, heterotrophic bacteria are more abundant in the hypolimnion than in the epilimnion (Niewolak 1974, Rheinheimer 1985) and that the maximum number of bacteria is observed when the temperature is in the range of 15° to 20° C (Jones 1977).

10. As would be the case in comparing soil in which terrestrial plants are growing with plant-free soil, the bacterial populations on aquatic plant surfaces are higher than in the water profile (Fletcher 1979), and individual aquatic macrophytes are capable of carrying varying quantities of epiphytic bacteria per unit surface area (Fry and Humphrey 1978). However, in contrast to observations of bacterial populations in the water profile, there is not yet an observable annual standard pattern of population dynamics of epiphytic bacteria (Fry and Humphrey 1978). Macrophyte-mediated changes in the littoral zone alter the metabolism and structure of epiphytic and surrounding phytoplanktonic communities (Godmaire and Planas 1986).

11. The various parts of the aquatic plant sustain different densities of bacteria (Wahbeh and Mahasneh 1984, Baker and Orr 1986). Aerial parts have lower bacterial populations than the submersed structures. Some species of aquatic plants have a significantly higher number of bacteria on the lower surface of the leaf than on the upper part, and none has as yet been described with more bacteria on the upper surface than the lower surface. The numbers of bacteria on the leaves nearest the apex are lower than those on the leaves further down the stem. These observations may reflect the influence of age on plant tissues (Baker and Orr 1986).

12. A significant amount of photosynthetically fixed carbon of submersed aquatic macrophytes can be secreted from various sectors of the plant. These dissolved organic materials are generally used by epiphytic bacteria before they reach the water column (Fry and Humprey 1978). Dissolved organic carbon compounds released during the decomposition of macrophytes can influence the composition and growth rate of the bacterial populations (Murray and Hodson 1986). Inhibitory compounds in leachates, e.g., flavones, phenolic acids, polyphenols, and soluble organic exudates (Wahbeh and Mahasneh 1984), act as selective agents that change the community structure of the bacterial population with respect to leachate resistance (Bastardo 1979, Planas et al. 1981, Godmaire and Planas 1986, Murray and Hodson 1986). Gram-negative bacteria, the dominant type associated with all plant tissues, decrease throughout the decomposition period, while the numbers of filamentous bacteria and anaerobic cellulolytic and gelatinous utilizer bacteria increase (Bastardo 1979).

13. In stagnant and anaerobic bodies of water, the dominant types of anoxygenic phototrophic bacteria are determined not only by the quantity and quality of light but also by the interaction within the bacterial community. Microaerophilic purple bacteria, Rhodospirillaceae, are unable to compete with anaerobic purple sulfur bacteria in the family Chromatiaceae in the presence of high concentrations of sulfide. At high concentrations, sulfide is toxic to the Rhodospirillaceae but promotes the growth of the Chromatiaceae. However, when acetate is the limiting substrate, Rhodospirillaceae can outcompete Chromatiaceae (Pfennig 1967).

14. In aerated areas of lakes, *Oscillatoria redekii* grows faster in the presence of heterotrophic bacteria than when grown in a pure culture. *Oscillatoria redekii* excretes nitrogenous compounds which, at higher

concentrations, are capable of inhibiting growth of the producer itself (Rheinheimer 1985).

15. Growth and feeding rates of protozoa increase with the concentration of bacteria (Sherr, Sherr, and Berman 1983). In areas of dense populations of epiphytic organisms, the productivity of submersed macrophytes may depend on the role of protozoa and other grazers (Rogers and Breen 1983).

16. Three important physicochemical factors that appear to influence the population of epiphytic bacteria and phytoplankton on aquatic plant surfaces are temperature (Baker and Orr 1986), light (Jones and Adams 1982), and nutrient availability (Hough and Wetzel 1975; Blotnick, Rho, and Gunner 1980; Carignan and Kalff 1982; Wahbeh and Mahasneh 1984; Godmaire and Planas 1986).

17. Management methods for the control of Eurasian watermilfoil based on the manipulation of biological systems have shown great potential advantages over other methods (Andres 1977, Schuytema 1977). A variety of organisms have been tested for use as biocontrol agents against *M. spicatum*. A snail, *Pomacea australis*, and the manatee, *Trichechus manatus*, were reported to be potent control candidates (Blackburn, Sutton, and Taylor 1971), but no further report has been given of their use (Amundsen and Brenkert 1978). Among 25 insects found to be associated with *M. spicatum*, *Paraponyx stratiotata* was the only one with sufficient host specificity to be considered for use as a biological control (Spencer and Lekic 1974). Two species of fish, *Tilapia zillii* and the grass carp or white amur (*Ctenopharyngodon idella*), have been used for aquatic weed management (Blackburn, Sutton, and Taylor 1971; Amundsen and Brenkert 1978). The inability of the former to survive low temperatures and the potential for the upset of ecological balances by the aggressive grass carp are significant drawbacks that have restricted the use of herbivorous fish as biological control agents (Bates, Burns, and Webb 1985; Blackburn, Sutton, and Taylor 1971).

18. Plant pathogenic organisms have also been tested for their ability to infect *M. spicatum* (Hayslip and Zettler 1973). Two fungi, *F. sporotrichoides* and *A. curvulum*, were reported to attack *M. spicatum* (Andrews and Hecht 1981; Andrews, Hecht, and Bashirian 1982; Patlak 1982). Although *F. sporotrichoides* can cause localized symptoms and *A. curvulum* can kill the plant under specific environmental conditions that increase plant susceptibility (Patlak 1982), the limitations on the effectiveness of these organisms

suggest that further study of the interactions of the plant, its microflora, and environmental conditions is necessary.

19. In the past, the manipulation of the rhizosphere has been used for plant protection rather than plant destruction. Koths and Gunner (1967) induced chitinase activity against *F. roseum* in an Arthrobacter species isolated from the rhizosphere of carnations. Subsequent dissemination of this chitinolytic bacterium in the rhizosphere of the carnations protected against *Fusarium* attack. The rationale for this treatment was that the original isolate, endemic to the rhizosphere, would, in effect, be sponsored by the carnation and helped to compete against other microorganisms in the environment. This hypothesis was confirmed by the isolation of approximately 65 percent of the original inoculum numbers by the time of plant harvest. Thus, plant-dependent microorganisms would appear to offer an ecologically attractive reservoir from which to draw in developing biological control strategies.

PART II: MATERIALS AND METHODS

Experimental Plant Material

20. *Myriophyllum spicatum* L. from Stockbridge Bowl, Massachusetts, was used in all experiments. For laboratory experiments, healthy tips were cut from plants in the field and brought back in lake water. Vegetative tips, 10 to 15 cm in length, were planted and allowed to form roots in aerated 10-gal (38-dm^3) aquaria illuminated with General Electric (GE) Gro-lux standard 40-W fluorescent plant lights, two lights per aquarium, for 8 hr each day.

Microbial Cultures

Fungal culture

21. *Mycoleptodiscus terrestris* (Gerdemann) Ostazeski (Ostazeski 1967) (*M. t.*), a cellulolytic organism originally isolated from necrotic areas on samples of *M. spicatum*, as previously described (Gunner 1983), was used throughout these studies.

Bacterial cultures

22. Eleven bacterial cultures were isolated from *M. spicatum*. These cultures were designated as MS11, P8, YC3, B10, B11, B12, B13, B14, B15, B16, and B17. Three isolated from *M. heterophyllum* were designated BHP, BSP, and MH2. All the cultures, except B17, were pectinolytic bacteria that were capable of forming a pitting colony (i.e., one that would form a significant trough in pectin agar medium as a result of vigorous pectinolytic action). Isolate P8, a gram-variable rod and spore former, was designated as *Bacillus* sp. (BSP8).

Culture Media

Cellulose agar (CA)

23. This medium was used for the maintenance of *M. t.* stock culture and for the formation of sclerotia by *M. t.* culture.

Martin's agar (MA) (Martin 1950)

24. This medium was used to enumerate fungal populations.

Pectin agar (PA)

25. This medium was used for the selection of pectinolytic microorganisms, for confirmation of their pectinolytic abilities, and for distinguishing between the strong pectin hydrolyzers (pitting colony) group and the mild pectin utilizing (nonpitting colony) group.

Polygalacturonic medium (PGM)

26. This medium was used for the induction of pectinolytic enzymes.

Potato dextrose agar (PDA)

27. This medium was used for the maintainance of fungal cultures and in the study of interactions among fungal populations.

Potato dextrose salt broth (PDSB)

28. This medium was used for the propagation of *M. t.* culture inoculum.

Sporulation medium (SPM)

29. This medium was used to induce the sporulation process of the bacterial cultures, including the BSP8 culture.

Trypticase soy agar (TSA)(BBL*)

30. This medium was used to maintain the bacterial cultures, to study interactions among heterotrophs, and to enumerate heterotrophic microorganisms.

Trypticase soy broth (TSB)(BBL)

31. This medium was used in propagating bacterial cultures in small batches. For larger volumes, TSB was prepared from the ingredients listed by Becton Dickinson and Company on their containers. Detailed descriptions of the media formulations (CA, MA, PA, PGM, PDSB, and SPM) are provided in Appendix A.

Cell Counts

32. Microbial populations were enumerated by the serial dilution method. Total heterotrophic bacterial counts were made on TSA. Macrocolony counts were limited to the heterotrophs grown on TSA that formed colonies which were larger than 1 mm in diameter, while microcolony counts were made of those 1 mm in diameter or less. Total pectinolytic bacteria were counted on PA. The pitting colony counts were based on the ability to excavate the medium surface

* Trademark of Becton Dickinson and Company.

within a period of 48 hr. All bacterial counts were made after 48 hr incubation at 28° C in darkness and presented as colony forming units (cfu) per gram dry weight of plant material or per millilitre liquid source. Fungal counts were made on MA after 96 hr incubation at 28° C and presented as propagules per gram dry weight of plant material or per millilitre liquid source. The majority counts of *M. t.* were derived from mycelial fragments; *M. t.* conidia were rarely observed.

Preparation of Inocula

Fungal inoculum

33. *Mycoleptodiscus terrestris* was propagated in PDSB (Gunner et al. 1985*). Small- scale batch cultures and fermentation starters were grown at 28° C on a rotary shaker at 125 rpm for 96 to 120 hr in Erlenmeyer flasks. Larger batch cultures, used in the 1985 field experiment, were grown in a New Brunswick Scientific Microferm 24-ℓ fermenter at approximately 28° C for 96 to 128 hr at an agitation speed of 250 rpm. For the 1986 field trial, the fungal cultures were propagated in a Pittsburg-Des Moines 40-ℓ fermenter at approximately 28° C for 128 hr. Each batch of the culture obtained was allowed to settle at 5° C in the dark, and the excess supernatant was discarded. The separate batches were blended in a Waring blender for 1 min (Gunner et al. 1985,* 1986*) and then pooled to provide a single concentrated culture prior to enumeration and inoculation. The fungal inocula for the 1987 field studies were grown at the Tufts University Biotechnology Engineering Center in a 300-ℓ Chemap fermenter at 30° C for 72 to 96 hr at an agitation speed of 300 rpm. The cultures were then passed through a Sharples unit at 13,000 g's to yield a cell paste. The paste was removed to sterile plastic bags, packed in ice during transportation, and stored at 5° C for up to 2 weeks. Prior to the inoculation, the cell paste (22.7 kg) was diluted to 250 ℓ in 3-ℓ batches. Each 3-ℓ batch, containing 273 g cell paste, was blended at high speed in a 4-qt (3.8-dm^3) Waring blender for 3 min before being distributed evenly among five 50-ℓ carboys.

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* H. B. Gunner et al. 1984-87. "Microbiological Control of Eurasian Watermilfoil," Annual Report to Aquatic Plant Control Research Program, US Army Engineer Waterways Experiment Station, Vicksburg, MS.

Bacterial inoculum

34. Bacterial inocula were grown in TSB.* Small batch cultures were grown in Erlenmeyer flasks at 28° C on a rotary shaker at 125 rpm for 18 to 36 hr. Large batch cultures were grown for 48 hr at room temperature in 15- to 18-ℓ carboys aerated with sterile air. The pooled batches of culture were enumerated before being mixed with 1-percent xanthan gum (Sigma Chemical Company, St. Louis, MO) prior to inoculation.

Visual Evaluation of Plants

35. The index of plant decline was assessed by visual evaluation of the experimental sites. The condition of the plants was rated on a scale of 0 to 3, with 0 indicating healthy growing plants and higher numbers representing the progressive decline associated with varying degrees of change in the following characteristics: internode elongation, discoloration, loss of turgidity, change in leaf structure, and new growth. The new growth, indicating plant recovery, was weighted against the rest of the categories by subtraction.

Specificity Testing

Pathogenic specificity toward nontarget aquatic and nonhost terrestrial plants

36. Four aquatic plants commonly found in New England lakes and ponds--*Vallisneria* sp., *Sagittaria* sp., *Elodea* sp., and *Lemna* sp. (obtained from Connecticut Valley Biological Supply Company, Southampton, MA)--were grown in aquaria and inoculated with 400 ml per aquarium of a solution of 1×10^7 cfu/ml of BSP8 in 1-percent weight/volume (1% w/v) xanthan gum and 200 ml per aquarium of a solution of 1×10^4 cfu/ml *M. t.* grown in PDSB. Plants were observed for 5 weeks after inoculation for pathological symptoms. Four common terrestrial plants were selected for screening of the pathogenic potential of *M. t.* Green bean *(Phaseolus vulgaris)*, pea (*Pisum sativum* L.), vetch (*Vicia* sp.), and wheat (*Triticum sativum*) plants were exposed to *M. t.* grown in PDSB by covering the plant surface with a suspension of blended mycelium.

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* H. B. Gunner et al., 1985, op. cit.

Plants were grown to maturity in a moisture chamber at ca. 90- to 100-percent humidity and observed for evidence of pathogenicity.

Pathogenic specificity to terrestrial plants identified as hosts*

37. Ten replicates of seedling and mature stages of red clover (*Trifolium pratense* L.), white clover (*T. repens* L.), three varieties of alfalfa (*Medicago sativa* L.), three varieties of bluegrass (*Poa pratensis* L.), oats (*Avena* sp.), and soybean (*Glycine max* L. Merr.) were inoculated with 2.1×10^5 cfu/ml of *M. t.* using a brush to ensure the presence of inoculum on the plant tissue. All the plants, treated and control, were grown in a moisture chamber illuminated with GE Gro-lux standard 40-W fluorescent plant lights. The numbers of susceptible plants, infected and dying, were determined at 2-week intervals for a period of 8 weeks.

Scanning Electron Microscope Studies

38. Samples were fixed with 2-percent glutaraldehyde in 0.1 M cacodylate buffer, postfixed with 1-percent OsO_4 in the same buffer and dehydrated through ethanol, and then critical point dried over liquid CO_2. Specimens were coated with gold-palladium in a Polaron Sputter Coater and examined with an ISI super III A scanning electron microscope (SEM).

Growth Curves of *M. t.* and BSP8

Growth curve of *M. t.*

39. A single sclerotium of a *M.t.* culture grown in CA and a 1-month-old mycelial culture grown on PDA were each transferred to PDA medium in petri plates and allowed to form mycelial cultures twice before use as a source of inoculum for the growth experiment. Cylinder-shaped agar blocks, approximately 0.4 mm in diameter and 0.5 mm in height, taken from the same radius of the *M. t.* colony were inoculated onto the center of four replicate agar plates

* See Gerdemann 1953, 1954; McVey and Gerdemann 1960; and Charudattan and Conway 1976.

containing PDA, PDSA, or MA. The diameter of the growth of each colony was measured daily and the average growth area of the colony determined.

Growth curve of BSP8

40. A culture of BSP8 was first propagated in SPM and TSB for 12 hr at 28° C. The population densities were then adjusted to an optical density, at 540 nm, of 0.01 in their respective media in a Spectrophotometer 21 (Bausch and Lomb). The viable count of BSP8 in the final concentration of TSB was 1.9×10^6 cfu/ml, and in SPM was 5.4×10^5 cfu/ml. Three-tenths millilitre of these adjusted concentrations was used to inoculate each of four replicates of 70 ml of the respective media in 300-ml nephelometer flasks. All the flasks, treated and untreated, were incubated at 28° C on a rotary shaker at 125 rpm. The optical density of each sample was determined at 2-hr intervals until the stationary phase of the growth in each medium was reached. The results obtained at each interval were averaged and plotted against time.

Microbial Interactions on Nutrient Media

Between *M. t.* and microbial populations from different sites on *M. spicatum*

41. Root systems from 10 randomly selected *M. spicatum* plants grown in aquarium culture were first aseptically separated from their respective stems; the remainder of the stem portions was then divided into two halves, the lower stems and the tips. Each group of tissues was suspended in 50 ml of sterile water and blended at maximum speed in a Waring blender for 5 sec. Following serial dilution, four replicates of dilutions at 10^{-1}, 10^{-3}, 10^{-4}, and 10^{-5} were spread on TSA, PA, PDA, and MA media, respectively. An agar block of *M. t.* culture was then placed in the middle of each plate before the plates were incubated at 28° C for 72 hr. The diameter of each colony was then measured, and the average of the four replicates was determined to assess differences in growth.

Between *M. t.* and microbial cultures from *M. spicatum* tissues

42. Four bacterial isolates (BHP, MS11, P8 (BSP8), and YC3) originating from *M. spicatum* were tested for their growth compatibility with *M. t.* Two-tenths millilitre of the stationary phase of each bacterial culture grown in TSB was spread onto TSA medium. Agar blocks of *M. t.* culture grown on PDA

were transferred to a designated area on each plate. After 48-hr incubation at 28° C, the diameter of growth of *M. t.* was measured, and any zone of inhibition exerted by the colony was assessed.

43. Fourteen bacterial cultures, isolated from *M. spicatum*, were point and streak inoculated onto the area at the edge of a 3-day-old *M. t.* colony grown on TSA, PA, PDA, and MA, respectively. After 48-hr incubation at 28° C, the growth of *M. t.* in the direction of inoculated and uninoculated areas was determined.

Growth response of bacteria exposed to phenolic compounds (Sigma Chemical Company)

44. The 14 bacterial cultures derived from *M. spicatum* were tested for their sensitivity toward 16 phenolic compounds detected in *M. spicatum* (Planas et al. 1981). Sterile Whatman No. 1 filter paper discs, 0.5 cm in diameter, soaked with 0.01, 0.1, 1, 10, 100, and 1,000 ppm of each phenolic compound, were placed on a lawn of bacteria on TSA medium. After 24-hr incubation at 28° C, the effects of the phenolic compounds upon the growth of the bacteria were determined. Cultures showing a response were tested again with the phenolic compounds at a concentration of 50, 500, and 1,000 ppm using the sensitivity plate method described above and the culture tube method containing TSB and the respective phenolic compounds at the same concentrations.

Jar Experiments

45. Five 10- to 15-cm tips of *M. spicatum* plants with roots were planted in 4 to 5 cm of a 3:1 mixture of sterile sand and soil in a plastic seedling cup. After overlaying the surface of the soil with gravel, the cup was lowered into a quart (0.9-dm^3) jar filled with distilled water. Plants in jars were maintained in an environmental chamber at 15° C on an 8:16 hr (light:dark) cycle.

Application of BSP8 and *M. t.* to *M. spicatum*

46. Three inoculum formulations were prepared for this experiment: control (untreated), *M. t.*, and BSP8 in 1-percent xanthan and *M. t.* The viable count of the blended culture of the *M. t.* grown in PDSB was 1.25×10^4 propagules/ml. Ten millilitres of *M. t.* was used to inoculate *M. spicatum* in the *M. t.* and *M. t.*-BSP8 experiments. The count of BSP8 grown in TSB was

1.5×10^7 cfu/ml. The cells were washed once by centrifugation using a Sorval RC2B at 10,000 g's for 25 min and resuspended in distilled water before 1-percent xanthan gum was added. Five millilitres of the BSP8-xanthan mixture was used to inoculate the plants prior to the addition of *M. t.* culture, as described above in the *M. t.*-BSP8 treatment.

47. Microbial populations in the water and on the plant tissues in each treatment jar were enumerated by the serial dilution method. A water sample was randomly taken from the jar before the plants were pulled out. The plants were suspended in 50 ml of sterile distilled water and blended at maximum speed in a Waring blender for 5 sec. The sample of blended plant suspension was immediately taken and used in the serial dilution process.

Dose response

48. To determine an optimum inoculation dose of *M. t.*, a series of jars were inoculated with 1, 3, and 5 ml of culture at a concentration of 3.5×10^5 propagules/ml. Each test was performed on quadruplicate plant samples. Jars were incubated at 18° C on an 8:16 hr (light:dark) cycle for 21 days.

Temperature response

49. To establish the optimum temperature for *M. t.* effectiveness, a series of jars were inoculated with 10 ml *M. t.* culture at a concentration of 4.4×10^5 propagules/ml and incubated at 18°, 21°, or 25° C, respectively, on an 8:16 hr (light:dark) cycle for 16 days.

Pool Experiment

50. Twelve 1.6-m^2 pools were planted with healthy *M. spicatum* tips. Thirty-six plants were placed in a 3:1 sand:pond soil mixture in 0.09-m^2 seedling trays 6 cm in height. Trays were placed in the semicircular half-pools at a depth of 65 cm after planting the tips. Plants were then allowed to root for 2 weeks prior to inoculation. Pools were filled with tap water and aerated vigorously until inoculation. The water was maintained at the same level throughout the experiment by the addition of fresh tap water as needed.

51. Prior to inoculation, the 12 pools were randomly assigned to four groups: control (no treatment), treated with BSP8 in xanthan carrier, treated with fungus *M. t.*, and treated with fungus *M. t.* and BSP8 in xanthan carrier. Each of the inoculum formulations described above was dispensed into the appropriate pool under low pressure through a spray manifold constructed of

1.3 polyvinyl chloride (PVC) pipe with eight 2 mm-aperatures. Pools treated with fungus received 500 ml of *M. t.* solution containing 2.6×10^4 cfu/ml, and pools treated with bacteria received 1.0 ℓ of inoculum containing 1.25×10^7 cells/ml in 1-percent xanthan. Pools receiving both treatments were treated with the bacterial inoculum first to allow attachment to the plant surface. Throughout the experiment, measurements were made of microbial populations, plant biomass, and plant condition.

52. Microbial populations were enumerated on both plant surfaces and in the water column for each treatment. Plant samples were randomly selected from designated trays, and water samples were taken from each pool on a weekly basis for enumeration of microbial populations. Visual evaluation of plant condition in each treatment was done at weekly intervals. Biomass data were collected weekly by the random harvest of five plants from each pool and at the termination of the experiment by measurement of total biomass in each pool.

Field Experiments

53. Field experiments were conducted at Stockbridge Bowl, in the town of Stockbridge, Berkshire County, western Massachusetts. The Bowl is a hardwater lake infested with *M. spicatum* in all areas less than 7 m in depth. *Myriophyllum spicatum* growth was continuously removed by a mechanical harvester from late spring to early fall.

Field experiment, 1985

54. An area, 2 to 2.5 m in depth, on the eastern side of the lake was chosen as an experimental site, and arrangements were made to prevent harvesting in the proximity of this 500-m^2 area. Eighteen 0.5-m^2 plots were marked with rectangular frames constructed of PVC tubing. Plots were randomly divided into two groups: control (no treatment) and treated with fungus *M. t.* and BSP8 in xanthan carrier. Inocula were prepared as previously described and transported to the field site in 45-ℓ carboys packed in ice. Plots were inoculated first with BSP8 in xanthan and then with *M. t.* through a spray manifold constructed of PVC tubing with sixteen 2-mm apertures. Inocula were delivered from a reservoir under 1.7 kg/cm^2 of pressure under the surface of the water in each treated plot. Treated plots were inoculated twice, once at the beginning of the experiment and again 8 weeks later. The first inoculum

consisted of 10 ℓ of BSP8 in 1-percent xanthan at a concentration of 7.7×10^7 cfu/ml and 4 ℓ of *M. t.* at a concentration of 1.35×10^4 cfu/ml. The second inoculum consisted of 10 ℓ of BSP8 in 1-percent xanthan at a concentration of 3.2×10^8 cfu/ml and 6 ℓ of fungus *M. t.* at a concentration of 1.2×10^4 cfu/ml. Measurements of microbial populations and visual evaluation of plant condition were carried out weekly. Microbial populations were measured on the plant tissue of samples taken from a designated plot for each treatment. Water grab samples were taken from all plots in each treatment and pooled prior to enumeration. Visual evaluations of plant conditions were made for each plot as previously described. Biomass was measured for plant material in the plots at the termination of the experiment.

Field experiment, 1986

55. Two sites, one treated and one control, 2 to 2.5 m in depth approximately 100 m apart, were chosen in the southeastern area of the lake. Each site, covering 15 m^2, was marked with an octagonal frame. The external part of the frame, which floated freely on the surface of the water, was constructed of 4-in. (10-cm) PVC pipe. A 4-mm clear plastic curtain, suspended from the external frame and reaching the lake bottom, allowed for better retention of the inoculum. An inner structure of 0.5-in. (1.3-cm) PVC pipe was inserted in the sediment to stabilize the floating frame and curtain surrounding the test site.

56. The inocula were prepared and transported as previously described. The first inoculation consisted of 125 ℓ of BSP8 in 1-percent xanthan at a concentration of 1.1×10^7 cfu/ml and 150 ℓ of *M. t.* at a concentration of 2.0×10^3 cfu/ml using a high-pressure sprayer previously described.* The unit delivered inoculum at the rate of 4 gal (15 dm^3)/min at 200 psi (1,380 kPa) through four spray nozzles held approximately 0.5 m below the surface. The second inoculation, 6 weeks following the first application, consisted of 125 ℓ of BSP8 in 1-percent xanthan at a concentration of 2.0×10^7 cfu/ml and 96 ℓ of *M. t.* at a concentration of 1.2×10^4 cfu/ml applied with a low-pressure spray manifold apparatus previously described* at pressures of ca. 5 psi (34 kPa) under the surface of the water.

57. Microbial populations on the plant surface and in the water profile were determined weekly for 14 weeks. The plant and water grab samples were

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* H. B. Gunner et al., 1985-86, op. cit.

collected randomly from each test site and transported in separate containers packed in ice. Microbial counts were performed on the day following sampling. Visual evaluation of the condition of the plants in the test and control sites was also carried out weekly for the same period. Biomass samples were randomly collected and measured at the termination of the experiment.

Field experiment, 1987

58. Two 70-m^2 sites, one treated and one control, 1.5 to 2.5 m in depth approximately 200 m apart, were chosen in the southeastern area of the lake. Test sites were marked off with buoys attached to cement blocks around the perimeters. The plants in these designated areas were allowed to grow without cutting throughout the experimental period.

59. The inoculum was prepared as previously described and transported to the lake in five 50-ℓ carboys packed in ice. Inoculation consisted of 250 ℓ of *M. t.* at a concentration of 6.5×10^4 propagules/ml applied using the spray manifold apparatus previously described* at a pressure of ca. 5 psi (34 kPa) just under the surface of the water.

60. Water and plant samples were collected weekly from experimental sites for 11 weeks to determine microbial populations. Water column samples were collected in sterile 250-ml polyethylene bottles from a depth of 1 m. Plants were divided into tips, midsections, and roots. Five 10-cm tips, five 20-cm portions from the plant midsection, and five entire root systems were collected from each site, put into sterile 250-ml polyethylene bottles containing 50 ml of sterile distilled water, and packed in ice for transportation.

61. Plant samples were processed upon returning to the laboratory by transferring the contents of each bottle to sterile 250-ml blender jars, blending at high speed for 5 to 10 sec, and making 10-fold dilution series of each. Samples were plated out and incubated as previously described. The remaining plant samples were dried at 105° C to a constant weight (48 to 72 hr).

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* H. B. Gunner et al., 1985, 1987 op. cit.

Biomass Harvest

Pool experiment

62. Trays were slowly lifted from the pools; spotters were present to catch any plant material that detached. The plants were removed from the trays by gently washing the soil mixture from their roots. The plants from each tray were then placed in a preweighed screen drying tray. The drying trays were placed in an oven at 105° C for 24 hr, removed, and weighed. They were then placed into the oven for an additional 12 hr and reweighed to ensure constant weight. The weight of the empty tray was then subtracted, and the biomass for each tray was recorded.

Field experiments, 1985

63. Biomass was determined for plants in all plots by cutting stems at the sediment level and bringing the plant material to the surface in wire baskets. Live and dead tissue were separated for measurement on the basis of differences in color and physical condition. For the purpose of this study, dead shoots were defined as those that were black or brown and had no green leaves emerging from them. All material was washed in tap water to remove marl; the material was then dried for 72 hr at 105° C in tared weighing screens.

Field experiments, 1986 and 1987

64. At the termination of the experiment, eight samples of the plant biomass were collected from each test site, each sample representing plant material from an area of 0.25 m^2. Samples were pulled from the marked area by a scuba diver. Excess sediment and foreign objects were washed from the plant samples before separation of stem and root sections. Each sample was oven-dried at 105° C until the weight remained constant.

Statistical Methods

65. Statistical analysis of data was performed using the F-test to determine overall variance and the t-test to determine if significant differences existed between treatments. Analysis was performed by computer application of BMDP statistical programs. The level of significance was determined within a 95-percent confidence interval ($P \leq 0.05$).

PART III: RESULTS

Growth Curves

Mycoleptodiscus terrestris

66. As shown in Figure 1, the growth of *M. t.* was highest on PDSA, slightly lower on MA than on PDSA, and lowest on PDA. The growth rates on each medium also differed accordingly.

BSP8

67. The growth curves of BSP8 in TSB and SPM are shown in Figure 2. Again, though rates of growth and yields differed slightly in the media tested, this may reflect the respective concentrations of inocula employed. The inoculum introduced to TSB was 5.7×10^5 cells, while that of SPM was 1.6×10^5, i.e., the inoculum of TSB was 3.5 times higher than that of SPM. This may then have been reflected in a lag phase in SPM that was 4 hr longer than in TSB. However, the stationary phase in TSB was at a higher level than in SPM, suggesting a more effective growth medium overall.

Microbial Interactions on Nutrient Media

Between *M. t.* and microorganisms from different sites on *M. spicatum*

68. As noted in Tables 1, 2, and 3, in all the combinations tested, the colonies of *M. t.* exposed to 10^{-2} dilution of the companion organisms were significantly smaller than those in 10^{-3}, 10^{-4}, and 10^{-5} dilutions and the control of *M. t.* grown by itself. No definite pattern of growth response was observed, and the maximum growth of *M. t.* was achieved in the presence of the respective accompanying organisms at concentrations ranging from 10^{-3} to 10^{-5}. In several instances, especially in the presence of organisms isolated from the plant tips (Table 1), the growth of *M. t.* colonies in low concentrations of microorganisms (10^{-4} or 10^{-5} dilution) was greater than the control. However, only *M. t.* grown on TSA medium lawned with 10^{-4} dilution from the tips was found to be significantly higher than the control. In general, the numbers of microorganisms recovered on PDA and MA media were lower than those recovered on TSA and PA media. Similarly, the growth of *M. t.* on PDA was more restricted in the presence of other organisms than with similar treatments on

the other media. On MA medium, the rate of growth of *M. t.* was similar in the control and treatments with low concentrations (10^{-3}, 10^{-4}, and 10^{-5} dilutions) of blended plant tissue suspensions.

Between *M. t.* and pectinolytic bacteria isolated from *Myriophyllum* sp.

69. To select a pectinolytic bacterium compatible with *M. t.*, inhibition studies were conducted to assess growth interactions. As shown in Tables 4 and 5, among the four candidate cultures tested, the highest growth of *M. t.* was observed on the plates lawned with bacterium strain MS11. However, MS11 showed the largest zones of growth inhibition by *M. t.* among the organisms tested. Agar blocks taken from the areas adjacent to *M. t.* colonies did not carry over any mycelium, since no growth was observed; however, these blocks were also capable of inhibiting the growth of MS11, as shown in Table 5. No inhibition of BSP8 was detected either by colony or agar block, and this was considered one necessary aspect for compatibility with *M. t.* Differences in colony size and zone of inhibition were observed in companion cultures when mycelia from different areas of the *M. t.* colony were employed.

Response of cultures to phenolic compounds produced by *M. spicatum*

70. Of 16 bacterial cultures from *Myriophyllum* sp. that were screened by the sensitivity plate method, five showed some observable level of response to the phenolic compounds tested. These five cultures were further examined for their reactions to phenolic compounds both by the sensitivity plate method and the culture tube method (broth media) (Table 6). The results obtained by the culture tube method were more sensitive than the plate method, though difficulty arose in reading results. In some cases, the interference resulted from the darkening of solutions by the mixture of phenolics and the TSB medium while, in a few instances, precipitate formed. In two cases, however, while growth was detected in the broth media, on the sensitivity plates small areas of inhibition were observed at the same concentration of phenolic compound tested. These were the treatments with bacterial strain BHP exposed to 1,000 ppm of m-coumaric acid and bacterial strain BSP in the presence of 1,000 ppm of syringic acid.

Population Dynamics of Plant-Associated Microorganisms in a Jar Environment

Application of BSP8 to *M. spicatum*

71. The experiments for which the results are noted below were conducted to assess the potential influence of a number of variables interacting with the populations of microorganisms on *M. spicatum*. These included the addition of xanthan, heat-killed cells, cells induced for the production of pectinolytic enzyme, and noninduced cells.

72. As shown in Figures 3-6, bacterial populations on plant tissues and in the water following the respective additions of xanthan, heat-killed BSP8 cells, and a mixture of xanthan and heat-killed cells were slightly higher than in the untreated samples. There was no significant difference in the populations of fungi in these control experiments (Figure 7).

73. Populations in the jars inoculated with the pectinolytic enzyme-induced culture were lower in the first 5 to 7 days than after inoculation with the noninduced culture (Figures 8-11). However, on the 15th day, this was reversed. Differing from the rest of the treatments, the pitting colonies in the treatment inoculated with enzyme-induced cells were the majority populations. The fungal populations resulting from enzyme-induced inoculum were slightly higher than the populations emerging after inoculation with non-induced culture (Figure 12).

74. With the addition of xanthan to both induced and noninduced cultures, the dynamics of bacterial populations were similar to those of the nonliving cell treatments (Figures 13-16). The pitting colonies in the enzyme-induced inoculum in the presence of xanthan still remained the major group among the bacterial populations recovered on PA medium. In the presence of xanthan, the fungal counts in the experiment resulting from inoculation with the enzyme-induced culture decreased on the 15th day (Figure 17).

75. No fungus was detected in the water of any of the treatments in this set of experiments.

Application of *M. t.* to *M. spicatum*

76. As shown in Figures 18 and 19, the heterotrophic bacterial populations were significantly higher, both on the plant tissue and in the water, than they were on untreated samples following the addition of PDSB medium to *M. spicatum*. However, the numbers gradually fell and approached the counts

of the heterotrophic populations in the control. Maximum differences were observed in the water, where up to a 10,000-fold difference in the populations was evident. There was a 10-fold increase in the number of heterotrophs on the plant tissue after PDSB was added; this difference did not persist and was no longer apparent by the termination of the experiment. Similar to the heterotrophic populations, following the addition of PDSB, the total number of pectinolytic organisms rose much more sharply in the water than on the plants (Figures 20 and 21). The population dynamics of the pitting colony showed a corresponding pattern to that of the total pectinolytics after the addition of PDSB.

77. In the treatments in which untreated culture supernatant or heat-treated supernatant was used as the inoculum, the dynamics of the heterotrophic (Figures 22 and 23) and pectinolytic (Figures 24 and 25) populations were similar to those in the PDSB treatment. The pitting colonies were even more closely related to the total pectinolytic populations than those observed in the PDSB treatment. In the water, the population dynamics of heterotrophic bacteria were different from those of the PDSB treatment; the heterotrophs in the supernatant treatment increased slightly by the second week, before dropping to the same level as the populations in the jars treated with heated supernatant. The pectinolytic populations in the heat-treated and untreated supernatant inoculated jars (Figures 24 and 25) stayed within the same range as the populations in the PDSB treatment. However, the pitting group and the total pectinolytic populations on the plant tissue (Figure 24) were, in both cases, more closely related than those observed in the PDSB treatment. The dynamics of the total pectinolytic populations in the water (Figure 25) were similar to those of the heterotrophs in the same treatment sequence, except in the heat-treated supernatant, in which the pectinolytic bacteria rose to two orders of magnitude above the number of heterotrophs present on the seventh day.

78. Following the treatment with either living or heat-killed *M. t.* mycelium (Figures 26-29), a virtually synchronous relationship was observed in all of the populations. The heterotrophic populations in the mycelium treatment (Figures 26 and 27) remained at almost the same level throughout the experiment. On the 14th day, a decrease in numbers of the heterotrophs was detected in the heat-killed mycelium treatment; then, on the 21st day, the number of heterotrophs increased to almost the same level as observed on the

seventh day. The dynamics of pectinolytic populations in the water of the mycelium-treated *M. spicatum* (Figure 29) were similar to those of the heterotrophic populations; however, the number of pitting colonies slowly decreased by almost two orders of magnitude within 21 days. The total counts of pectinolytic and pitting colonies in the water profile of the heat-killed mycelium treatment exhibited a 100-fold decrease in populations on the 14th day while, on the plant tissue (Figure 28), a slight increase was observed in the same period of time.

79. When the live *M. t.* mycelium suspended in untreated culture supernatant and heat-killed mycelium suspended in heat-treated supernatant were used as the inocula, the dynamics of the total heterotrophic populations either on plant tissue (Figure 30) or in the water (Figure 31) were closely related. The heterotrophic bacterial populations that emerged after inoculation with the heat-treated mixture of *M. t.* mycelium and supernatant were slightly higher than those inoculated with the live mycelium and untreated supernatant combination in both plant tissue and water profiles. The total pectinolytic counts and pitting colonies in the live mycelium-supernatant treatment rose in the water (Figure 32); however, the populations on the plant tissue (Figure 33) remained stable or decreased slightly. The total pectinolytic populations in the heat-treated combination in the water (Figure 32) remained stable for 2 consecutive weeks before decreasing on the 21st day. However, the number of pitting colonies stayed at relatively the same level throughout the experimental period. On the plant tissue receiving the heat-treated inocula, the total pectinolytic bacteria slowly decreased, and the majority of the pectinolytic bacteria on the 14th day were of the pitting type.

80. The population dynamics of fungi are shown in Figures 34 and 35. Only in the live *M. t.* mycelium treatment and in combination with supernatant did the fungal populations increase to an observable level. On the plant tissue, the predominant fungus observed was *M. t.* (Figure 34), and the differences in the dynamics of the populations were minimal. Similar to the situation on plant tissue, the dominant species of fungus in the water of the mycelium treatment was *M. t.* (Figure 35), while *M. t.* exhibited its ability to become the dominant species in the water in the live mycelium-supernatant treatment.

Application of BSP8
and *M. t.* to *M. spicatum*

81. Total counts of the various groups of microorganisms selected for monitoring were delineated by their respective growth criteria, and each group became dominant at a different period. The population dynamics of the major types of colonies (e.g. macrocolonies and microcolonies on TSA medium, pitting and nonpitting colonies on PA medium) in part exhibit the interactions of microorganisms on the plant surface and in the surrounding water.

82. The numbers of heterotrophic bacteria on the untreated plant tissue recovered on TSA were approximately 100-fold higher than the counts in the surrounding water (Figures 36 and 37). On the plant, microcolonies became dominant in the latter period of the experiment (after 30 days). In contrast, macrocolonies were dominant in the water during the same period of time. The pectinolytic bacteria on the plant tissues were also 100 times more numerous than in the water (Figures 38 and 39). The nonpitting colonies were the dominant type in the water throughout the experimental period. However, on the plant tissue, nonpitting colonies were the dominant form during the first 10 days and from the 24th day on. The strong pectin hydrolyzing bacteria in the water were at least 10-fold lower in numbers than on the plant tissue or the nonpitting populations in the same environment. A similar situation was also observed with fungal populations. The fungal counts on the plant tissue were at least an order of magnitude higher than the counts in the water (Figures 40 and 41). At low concentrations, a few colonies of *M. t.* were observed on plant tissue, but none were detected in the water.

83. After the addition of *M. t.* inoculum, the counts of heterotrophic bacteria from both plant tissue and water (Figures 42 and 43) dropped 10-fold below the counts determined in control jars (Figures 36 and 37). Within a period of 7 days on plant tissue, and 10 days in water, the populations rose to a level 10-fold higher than those in the control jar. The bacterial counts in the water remained high for 10 days before a sharp decrease in populations was detected, subsequently remaining close to the level observed in the control. The populations on the plant tissue remained high for the next 30 days before declining to the levels detected in the control. The macrocolonies and microcolonies showed no distinguishable domination of one over another in either the water or on plant tissues, except during the period between 3 and 17 days when the macrocolonies established their domination.

Differing from the counts on TSA and those from the control, the nonpitting colonies on PA were the dominant group in this *M. t.*-treated set of the experiment. The total population dynamics on TSA (Figures 42 and 43) and PA (Figures 44 and 45) media were similar, though the decrease in microbial counts in the final period of the experiment on PA was not as persistent as on TSA. The fungal populations recovered on MA following the inoculation with *M. t.* (Figures 46 and 47) were approximately an order of magnitude higher than in the control. The *M. t.* population was capable of competing with other fungi on the plant surface for a period of 41 days before its decline was observed. Differing from the numbers in the control, the fungal counts in the water, following a sharp drop in the first few days, remained at almost the same level throughout the experimental period. The numbers of *M. t.* recovered from the water varied widely, though a similar pattern of decline of the *M. t.* in the water and on the plant tissue was observed.

84. With the addition of the BSP8 in 1-percent xanthan and the *M. t.* culture, the total number of heterotrophs on *M. spicatum* tissue (Figure 48) remained almost at the same level throughout the experimental period. This level was approximately 100-fold greater than the numbers detected on the untreated plant tissue and at least 10-fold higher than in the water of the same treatment. During the first few days, the heterotrophic bacteria in the water (Figure 49) were up to three orders of magnitude higher in number than in the control. Subsequently, the populations decreased to almost the same level as those detected in the control. As in the *M. t.* treatment, the macrocolonies were the dominant group during the first part of the experiment. However, during the final 2 weeks, the microcolonies dominated both on plant tissue and in the water.

85. The population dynamics of pectin utilizers on the plant tissue (Figure 50) were similar to those of the heterotrophs in the same treatment. In the water (Figure 51), however, the fluctuations of the pectinolytic populations were greater than those of the heterotrophs, and a similar trend of decline could be observed. As in the *M. t.* treatment, the nonpitting colonies were the dominant group in the dynamics of the total pectinolytic bacteria.

86. The populations of the pitting and nonpitting colonies in the BSP8-*M. t.* treatment of both plant tissue and the water were higher than those in the *M. t.*-treated experiment. The difference between the populations on

the plant tissue was not as great as in the water. For the first 10 days of the experiment, *M. t.* was found to be the dominant fungus on the plant tissue (Figure 52), and also for the first 3 days in the water profile (Figure 53). After the 10th day and for most of the remainder of the experiment, the population of *M. t.* in the water dropped below the detectable level. In the presence of both BSP8 and *M. t.*, the population of *M. t.* on the plant tissue was lower than the populations in the experiment in which *M. spicatum* was treated with *M. t.* alone.

Visual Evaluation of the Response of *M. spicatum*

Applications of BSP8 formulations

87. No significant changes in the condition of *M. spicatum* were observed during the 15 days of the experiment described above. The response of *M. spicatum* to extended exposure (30 days) to BSP8 was observed as internodal elongation generated by the presence of the bacterium.*

Applications of *M. t.* formulations

88. As shown in Figure 54, different rates of *M. spicatum* decline were observed following treatment with various regimens of PDSB culture medium. The rate of decline of the plants treated with sterilized PDSB increased after the 13th day and reached the severe level in the following 6 days. The rate of decline of the plants in the treatment with the sterilized culture supernatant increased after the 13th day and remained higher than that obtained with the application of the untreated supernatant. The index of plant decline of the untreated *M. spicatum* remained low and virtually the same throughout the experiment.

89. The plant decline indexes following the treatment with heat-killed and live *M. t.* mycelium, and with the addition of heat-treated and untreated supernatants, are shown in Figure 55. The rate of plant decline was faster in the presence of live *M. t.* mycelium, whether applied alone or together with untreated supernatant, than in the other applications. The most effective treatment was the mixture of live mycelium and untreated supernatant, in which the indexes of plant decline reached the severe level in the shortest time. The decline rate of *M. spicatum* following the treatment with live *M. t.*

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* H. B. Gunner et al., 1985, op. cit.

mycelium was slightly lower than with the live mycelium-untreated supernatant mixture, but higher than the rest of the treatments. Heat-killed mycelium in the presence of heat-treated supernatant had a slightly more severe effect upon plant decline than the heat-killed mycelium alone. However, on the 23rd day, the accelerated degree of plant decline of the former was approximately two indexes higher than the latter.

Applications of BSP8 and *M. t.*

90. In Figure 56 are shown the indexes of *M. spicatum* decline following treatment with either *M. t.* alone or BSP8 and *M. t.* together. The untreated plants declined slowly, until the 42nd day of the experiment; the rate then increased slightly and remained at the same level until the termination of the experiment. In the treatments with *M. t.* and the combination of *M. t.* and BSP8, the respective degree of plant decline was significantly higher than in the control. The plants in the *M. t.* and BSP8 treatment exhibited a slightly higher level of decline than those exposed to *M. t.* alone; however, at the termination of the experiment, complete plant decline was observed in both treatments.

Dose Response

91. *Mycoleptodiscus terrestis* used alone as a control agent is capable of inducing *M. spicatum* death (Figures 57 and 58). Inoculation concentrations of 1 and 3 ml produced increasing levels of plant decline, while 5 ml resulted in complete degradation. Not only does viable plant tissue decrease (Figure 57), but the extent of root development diminishes (Figure 58) with increasing inoculum levels. The results of biomass determinations (Figure 59) support the visual evaluation, showing a decrease in plant dry weight with increasing inoculum levels. Though there was a 10-percent decrease in plant biomass as the inoculation dose rose from 1 to 3 ml, plant weight decline did not continue at the 5-ml dose level.

Temperature Response

92. *Myriophyllum spicatum* response to inoculation with *M. t.* also appeared to be conditioned by water temperature. As shown in Figure 60, when water temperatures increased from 18° to 25° C, the *M. t.* efficacy, as

measured by decrease in plant weight, rose. This was confirmed by the significant decrease in biomass weight between treated and control trials at 25° C (P value, 0.047), while no significant difference between treated and control plants occurred at either 18° or 21° C.

SEM Examination of *M. spicatum* Treated with *M. t.* and BSP8

93. On the 21st day of the BSP8-*M. t.* experiment, representative *M. spicatum* tissue from each treatment was prepared for examination by SEM.

94. The microorganisms observed on the surface of the control leaves and stems, young and old (Figures 61-64), were virtually all bacterial populations. No fungi were observed on the samples selected.

95. On the surface of *M. spicatum* inoculated with *M. t.* (Figures 65-68), fungal mycelia were observed together with slightly denser bacterial populations, as well as a number of amorphous structures.

96. The maximum accumulations of bacterial populations on the surface of *M. spicatum* were observed in the treatment with the BSP8-xanthan mixture and *M. t.* as the inocula (Figure 69-72). The weblike structures deriving from the xanthan appeared to trap the bacteria and component parts of the fungal mycelium on the plant surface.

97. In Figure 73 is shown the propagule from which the infectious mycelium originated. The penetration of *M. t.* mycelium into *M. spicatum* tissue is shown in Figure 74.

Pool Experiment

98. At the termination of the experiment, control pools yielded plants that were generally representative of prevailing seasonal conditions, i.e., the period of decline, when new tips are produced while old stems decay to allow release of this new growth. Root structures remained intact presaging regrowth of the plant in the new season.*

99. Plants from pools inoculated only with the pectinolytic BSP8 showed the internodal elongation of stems consistent with the jar experiments. It

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* H. B. Gunner et al., 1985, op. cit.

was further apparent that there was a delay in seasonal decline compared to that in control pools. This was expressed by a reduction in stem necrosis and greater amounts of green tissues with proportionately fewer tips.

100. The application of the fungus to *M. spicatum* was observed in pools where the direct impact of the fungal attack was seen in the widespread occurrence of necrosis and the lack of new tips and green material. Root tissue could not be recovered due to its severance from the decayed stems at the time of harvest.

101. The effects of treatment with bacterium and fungus together were evident in pools where stem elongation was again observed. There was a virtual absence of new growth and severance of roots at plant harvest.

102. In Figure 75 are shown the results of visual evaluations of changes in the appearance of pool-grown plants. Under these conditions, the emergence of extensive epiphytic algal populations in all groups hastened the decline of control plants and minimized the difference observed until the plants were actually harvested. Although the visual evaluations showed a general decline in disease index as evidenced by new tip growth, these data did not reflect the significantly reduced biomass in pools treated with fungus alone or fungus with BSP8.

103. Analysis of biomass data (Table 7) showed that the biomass harvested from the control pools was significantly greater than that harvested from pools treated with the fungus alone or with fungus and bacterium together. Treatment with the bacterium alone did not result in any significant loss of biomass with respect to the control pools. The presence of the bacterium, even when added to the fungal inoculum, did not result in any significant loss in biomass in comparison to the treatment with the fungus alone. Comparison of the biomass from the groups treated with the fungus and the combination of bacterium and fungus showed no significant difference between the two treatments.

104. As can be noted in Figures 76 and 77, the total numbers of microorganisms recovered on TSA from plant tissue or from the water profile remained constant in their relationship throughout the various treatments. Inoculation did not result in any observable shift in microbial numbers, although an overall increase in colonization of the plant tissue emerged as the experiment progressed. Total numbers in the water column were approximately three orders of magnitude lower than on the plant tissue.

105. Examination of data on population changes induced by the addition of the fungal and bacterial inoculation revealed that the addition of these organisms did not result in a residual increase either on the plant tissue or in the adjacent water profile. Numbers in the water column were lower than those on the plant tissue and showed a greater variability.

106. Similar to the above, the numbers of strongly pectin hydrolyzing (pitting) bacteria were up to four orders of magnitude higher on the plant than in the water (Figures 78 and 79). The counts both in the water and on the plant tissue of the control slowly changed throughout the experiment, first declining and then slowly returning to near initial levels. A similar pattern of gradually changing numbers was detected for the strongly pectinolytic bacteria in the water of the pool treated with BSP8 and *M. t.* The population dynamics in either BSP8-treated or *M. t.*-treated pools were unstable; however, parallel patterns of counts were observed after the fourth week of the experiment. The numbers of strong pectin utilizers on the plant tissue of the treated pools were generally higher than on the control. Distinguishable differences were observed at the second week and the fourth week. At the second week, the counts on the tissue treated with BSP8 and *M. t.* were more than 10-fold greater than the others, and at the fourth week the pectinolytic populations on all of the treated pools were almost 10-fold greater than the control.

107. The populations of fungi on the plant tissue were up to five orders of magnitude higher than in the water profile (Figures 80 and 81). The difference in fungal counts on the plant tissue of all the treatments including the control stayed within the range of one order of magnitude, except on the first week after inoculation when almost 1,000-fold differences were observed between the count in *M. t.*-treated pools and over the controls. In the water, the numbers of the fungi detected were very low, especially in the *M. t.*-treated pools, in which only a few colonies were observed in plate counts during the last 2 weeks of the experiment. However, in the BSP-8 and *M. t.*- treated pools, similar low numbers were determined throughout the experimental period, except at the sixth week, when none of the fungus was detected in any control or treated pools.

Specificity Tests

Selected aquatic and terrestrial plants

108. As will be noted in Table 8 in the preliminary trials, no evidence of infection was obtained from a variety of plants, both aquatic and terrestrial, inoculated with *M. t.*

Established terrestrial hosts

109. The responses of an additional number of terrestrial plants to infection by *M. t.* are shown in Table 9. Even in those plants that were previously identified as hosts of *M. t.*, a high level of inoculum (2.1×10^5 cfu/ml) was required to elicit significant symptoms of infection. While the seedling stage of two species of clover, three varieties of alfalfa, and one variety of bluegrass were sensitive to infection by *M. t.*, their mature counterparts showed no significant difference between treated and control plants. In contrast, seedlings of oats and two other varieties of bluegrass showed a higher level of tolerance to infection than did their mature stages. No significant effect was observed on soybean seedlings or mature plants.

Field Experiments

Field experiment, 1985

110. The response of *M. spicatum* in Stockbridge Bowl to the application of the microbial agents *M. t.* and BSP8 was observed 2 weeks after the second inoculation.* The leaves were necrotic and the stems bleached; no green tips were observed. In an untreated plot, the plants were green; there was little necrosis, and healthy growing tips were plentiful.

111. Biomass determination (Table 10), at the termination of the experiment, did not show significant differences between treated and untreated plots.

112. Analysis of microbial population dynamics on plant tissue and in the adjacent water profile revealed that increases in microbial numbers were generally transient (Figures 82 and 83). Increases in numbers reflected the introduction of inoculum and were only briefly sustained. As the plant declined, there was a sustained peak of microbial populations. However,

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* H. B. Gunner et al., 1985, op. cit.

visual evaluation of the plant response to inoculation (Figure 84) showed that observed plant decline was coincident with maximum microbial numbers on the plant surface. It will be noted that the maximum decline followed the reinoculation of the plant with *M. t.* and BSP8. Figures 85 and 86, in which are shown the counts of pectinolytic bacteria on plant tissue and in the water profile, reflect the greater stability of numbers on plant surfaces. Although numbers were significantly greater subsequent to inoculation, they declined to baseline levels not significantly higher than in the controls.

113. Fungal numbers (Figures 87 and 88) remained relatively stable on plant tissue after inoculation but declined sharply in the water column to almost undetectable levels. However, counts of both fungal and pectinolytic bacterial populations on the plant tissue rose sharply after the second inoculation before declining to near control levels.

Field experiment, 1986

114. Visual evaluation of *M. spicatum* (Figure 89) subsequent to the first inoculation showed a slightly higher degree of deterioration in treated plants than in control plants. The decline elicited by the second inoculation in the treated site, however, was up to five orders of magnitude higher than in the control, clearly indicating a very high degree of physiological disruption in the treated plants. Within the degraded tissue at the bottom of the treated site, a few young tips were present, but none were visible from the surface of the water even at the termination of the experiment (Figure 90). The decline observed initially in the control site was dictated by the natural physiological cycle of the plant; however, no further significant visual changes were observed after the seventh week of the experimental period (Figure 91).

115. The effects of treatment on the biomass of Eurasian watermilfoil are shown in Table 11. An approximate 16-fold reduction of the stem-leaf biomass and a 10-fold decrease in root biomass were observed in the samples taken from the treated site compared with those from the control. The biomass of stem-leaf tissue in the control site was 26.7 times greater than its root biomass, while it was only 17.2 times higher in the treated site.

116. It will be noted from Figures 92 and 93 that the total numbers of bacteria isolated from *M. spicatum* tissues remained three to four orders of magnitude higher than the numbers observed in the adjacent water profile. Total bacterial counts from the water profile reflected virtually a steady

state throughout the experimental period (Figure 93). The one perturbation evident occurred following the second inoculation; however, the additional numbers of bacteria fell to baseline levels within 3 days. The bacterial counts from the plant tissue gradually increased following the first inoculation. The second inoculation sustained numbers at a somewhat higher level.

117. The numbers of strongly pectinolytic bacteria in the water profile from plant tissue (Figures 94 and 95) showed distribution patterns similar to those of the total bacterial counts in that the numbers on plant tissue were significantly higher. Although the control tissue showed a higher level of strongly pectinolytic bacteria at the onset of the experiment, following the application of the first inoculum, the populations on the treated tissue increased to levels higher than those of the controls and rose even more sharply subsequent to the second inoculation.

118. Fungal population numbers (Figures 96 and 97) were low in the water profile, in some instances dropping below the detectable level of the plate count method employed. The differences of the fungal counts taken from the control and the treated plant tissue were minimal, except during the sixth and seventh weeks, when a significant drop in fungal numbers was observed in control samples, and on the tenth week after the second inoculation when the treated samples showed approximately a 10-fold increase in fungal propagules over the control tissue. *Mycoleptodiscus terrestris* was recovered from the treated samples following the first inoculation and throughout the course of the experiment.

Field experiment, 1987

119. The effect of treatment with *M. t.* on *M. spicatum* as measured by biomass decline is shown in Table 12. A 71-percent reduction in total plant biomass was observed, along with a fold reduction in stem-leaf biomass and a twofold reduction in root material. The biomass of stem-leaf tissue in the control site was 15.3 times greater than its root biomass, while it was only 7.4 times higher in the treated site.

120. The numbers of total heterotrophic bacteria isolated from various *M. spicatum* tissues and from the adjacent water profiles are shown in Figures 98-101. The bacterial numbers from plant tissues remained four to five orders of magnitude higher than those from the water profiles, with root numbers an order of magnitude higher than tip and midsection numbers. Bacterial counts from the plant tips and water profiles remained virtually at

a steady state throughout the experimental period (Figures 98-101). Bacterial numbers from the treated plant midsection increased during the second week after inoculation (Figure 99) and remained close to an order of magnitude higher than control numbers for 4 weeks before declining. Root populations (Figure 100) were not only one order of magnitude higher than tip and midsection populations, but also showed a distinct pattern of fluctuation not evident in other plant or water populations.

121. The numbers of strongly pectinolytic bacteria from plant tissues and water profiles (Figures 102-105) showed distribution patterns almost identical to those of the total heterotrophs in terms of population levels and stability. Strongly pectinolytic bacteria on the midsection of treated plants increased from a low at week 5 (Figure 103) to a maximum at week 8. Indeed, the fourth week after inoculation, they remained significantly higher than control numbers for 4 weeks before declining to control levels.

122. As shown in Figures 106-109, the numbers of total fungi on plant tissues and in the water profiles followed the same general pattern shown by bacterial populations in that plant tissues supported populations three orders of magnitude higher than those found in the water profiles. Total fungal populations were, however, 1 to 2.5 orders of magnitude lower than the corresponding bacterial populations. In contrast to bacterial populations, total fungal populations from plant tissues were stable throughout the experimental period, most notably root populations, while water profile populations fluctuated considerably (Figure 109).

123. The numbers of *M. t.* isolated from the plant tissues during the lake trials are shown in Figures 110-112. At no time during the experimental period was *M. t.* observed in the water profile. *Mycoleptodiscus terrestris* was observed sporadically on control tissues, most frequently on midsections. It did occur after inoculation on treated tissues. In contrast to control numbers, treated tips (Figure 110) showed sustained *M. t.* populations over the 4-week period immediately following inoculation, after which they declined to an undetectable level.

PART IV: DISCUSSION

124. Throughout the work reported, growth of the selected cultures both individually and in concert was observed to be critically conditioned by the nature of the nutrient medium employed. It was established early that, for *M. t.*, PDSB was the growth medium of choice, and TSB gave maximum growth for BSP8.

125. When microbial interactions were observed on various nutrient media, it was significant that additional elements apart from the growth media characteristics entered into determining the level of growth achieved. These included the specific sites from which isolates were derived, i.e., tip, stem, or root, and the respective concentrations of interactive microbial populations, regardless of site or origin. Thus, the highest populations of bacteria were invariably recovered on TSA and PA rather than on PDA and MA. This would suggest the preponderance of bacteria with complex nutritional needs, on the one hand, and of pectin utilizers on the other. It also reflected the relative paucity of fungi both on the plant surface and in the adjacent water profile. If the interaction of bacterial populations and *M. t.* is considered, bacterial populations at relatively similar concentrations promoted the growth of *M. t.* regardless of the sites of isolation. Bacterial cells isolated from the tip (Table 1) and stem (Table 2) of the plant exercised a stimulatory effect on the growth of *M. t.* when their higher numbers would have suggested competitive inhibition; thus, 10^{-4} cells from the tip and stem achieve the same stimulus to growth as 10^{-5} cells from the root (Table 3). It was subsequently also reflected in the more active plant response to *M. t.* inoculation to the growing plant tip than to applications to other areas of the plant. This phenomenon was restricted to TSA medium and did not occur on PA, suggesting a more competitive relationship between *M. t.* and the pectinolytic microflora. This was further shown in the relationship between *M. t.* colony size and the number of pectinolytic bacteria on solid medium. The competitive interaction that was observed with *M. t.* was largely with bacteria, reflecting the low number of fungi present. However, fungal populations recovered on MA showed no distinguishable competition with *M. t.*

126. Critical to the control strategy was the characterization of pectinolytic bacteria association with *M. spicatum* and the establishment of their compatibility with *M. t.* It was therefore of interest that a growth

progression could be established denoting the enhancing or inhibiting effects of the various pectinolytic isolates. In descending order of preference, *M. t.* grew better in the presence of MS11, BHP, BSP8 (P8), and YC3. On the other hand, *M. t.* exhibited inhibitory pressure on the pectinolytic bacteria which, in descending order, included MS11, BHP, YC3, and BSP8. Thus, BSP8 was both mildly stimulatory to *M. t.* and entirely compatible as a companion organism for the control strategy. That the nature of the interaction between *M. t.* and the pectinolytic isolates was a complex one was reflected in the variety of inhibitory phenomena observed. Thus, an agar block excised at a distance of 1 mm beyond the visible limit of a *M. t.* colony could inhibit growth of MS11 but not of the other bacteria tested, indicating a diffusible excretion by the *M. t.* with a specific and delimited range of activity. An additional variable in these interactions was the age of the *M. t.* mycelium. Older mycelium was more inhibitory to bacterial growth than younger; while younger mycelium grew better in the presence of BHP, BSP8, and YC3, slightly older mycelium grew best in the presence of MS11. In general, it would appear that younger mycelia are metabolically more active and accumulate lower concentrations of toxic substances than older mycelia, and that such toxic substances are specific in their range of action.

127. A further question requiring resolution before adopting specific microorganisms for the biological control of *M. spicatum* was the capacity of such an organism to withstand the phenolic compounds known to be associated with *M. spicatum*. These compounds are known to elicit a differential response among various microorganisms. Again, microbial response to the phenolic compounds tested was confounded by the nature of the medium employed. Broth medium containing phenolic compounds showed more inhibitory effects than similar exposure on agar plates. The most toxic effects were observed in TSB, presumably reflecting the more direct contact between the phenolic compound and the cells compared with the restricted diffusion on the agar plate. Among the organisms tested, in descending order of sensitivity, were MS11, BHP, and BSP. Ellagic, gallic, tannic, and 3,5-dimethoxy-4-hydroxycinnamic acid, which have been reported to be present in *M. spicatum* at a higher concentration than other phenolics (Planas et al. 1981), inhibited the growth of bacteria tested more effectively than the remaining phenolics tested. These results support the possibility that phenolic compounds produced by the plant act both as a

defensive chemical and as a selective agent for the microbial populations associated with the plant surface and the adjacent water profile.

128. In preliminary experiments conducted in jars to assay the effects of the potential microbial control agents, a number of observations remained constant; first, after applications of both BSP8 and *M. t.*, at the outset and throughout the experiment, numbers of microorganisms were consistently higher on the plant tissues than in the adjacent water. This was true as well in untreated controls, confirming that population dynamics were nutritionally determined by the plant. Where inoculations of BSP8 and a *M. t.* were made, numbers rose accordingly, reflecting the compatibility of these organisms with *M. spicatum* from which they had originally been isolated.

129. It should be noted that the addition of any nutrient medium, e.g., xanthan, supernatant, etc., served as a general stimulus to microbial proliferations. Nonetheless, the addition of BSP8 resulted in a remarkable dominance by this organism over other bacteria present. It may be speculated that this was the result of two processes: first, the affinity of this bacterium for specific sites on the plant surface and, second, the subsequent release by pectinolytic action of newly available substrates for the successive growth of fresh populations. Added to these factors could be the selective pressure exerted by the plant through its phenolic constitution, via induced resistance and/or the unavailability of attachment sites to microorganisms foreign to the plant ecosystem.

130. It must be stated that, in the recovery of BSP8, although no distinction was made between BSP8 and other strongly pectinolytic microflora recovered, the dominance of the BSP8 colony type appeared sufficient to confirm that this was indeed the preeminent pectinolytic species present. The BSP8 colony type was not observed in the uninoculated controls. Further confirmation for the dynamics of pectinolytic microfloral change proposed above is provided by the numbers of pectinolytic organisms that were consistently recovered from treated plants and the adjacent water. Although numbers of nonenzyme-induced populations appeared initially higher, they invariably fell to a level below enzyme-induced inoculum. The addition of xanthan as a carrier for BSP8 inoculum appeared to exert only a minor influence on the population dynamics; however, as will be discussed more fully below, subsequent observations with the SEM revealed that the xanthan did appear to provide an adhesive matrix for the enhanced settling of cells on the plant surface.

Fungal numbers remained consistently low in the jar bioassay, undoubtedly reflecting low nutrient availability in this setting and the competitive handicaps placed on the fungus in this constrained environment. However, the fungus was inevitably present on the plant and successfully reisolated from it even though it was seemingly absent in the water. The addition of BSP8 in a variety of formulations did not seem to significantly affect fungal numbers.

131. As previously noted, the application of *M. t.* in the jar bioassay, as with BSP8, resulted in higher numbers of organisms on the plant tissues than in the adjacent water. This would suggest again the more immediate availability of nutrients on the plant surface. An inconsistent pattern of response by the bacterial populations to the addition of *M. t.* was observed. The addition of PDSB medium alone, or of the heated and unheated supernatant of *M. t.* culture, stimulated little change in the pattern of bacterial growth observed. However, the presence of *M. t.* mycelium in these preparations, whether live or killed, enhanced changes in bacterial population dynamics. Indeed, presumably by releasing metabolic products only scantily available from the live mycelium, the heat-killed mycelium induces an even sharper effect. The changes observed were most pronounced in the second week of the experiment. This would suggest a period of population equilibrium in which the bacteria compatible with *M. t.* became dominant.

132. A further observation of interest was that as numbers of strongly pectinolytic organisms increased, so did the total number of pectinolytics present. Apparently, the decomposing activity of the pectinolytic flora made progressively more nutrients available from the plant tissue, thereby stimulating additional population growth. The recovery of *M. t.* from both plant surfaces and the water profile confirmed that its presence was significant in establishing bacterial populations in the jar setting. The major component of the fungal population isolated from plant tissue treated with *M. t.* mycelium was, in fact, *M. t.*, and the same held true for the isolations made from the water. However, early isolations from the water profile revealed the presence of other fungi, and only after 7 days was *M. t.* isolated, eventually to become the dominant fungal species. This further confirmed the determining effect of the plant in *M. t.* population dynamics, as *M. t.*, supported by the plant, subsequently made its way into the water profile.

133. In the attempts to more closely monitor population changes, the dominant forms present were identified as pitting and nonpitting pectinolytic

colony formers, macrocolony and microcolony formers, and *M. t.* and other fungi. The inoculations with *M. t.* and BSP8 were closely observed for their effects in shifting the population equilibria of the above groups. A further element in population ordering was the decline of *M. spicatum* and the release of nutrients as well as inhibitory substances during this process. The results observed reflect constantly shifting patterns in which the major lever for change was the introduction of either BSP8 or *M. t.* cells. The introduction of BSP8 stimulated the appearance of both pitting as well as nonpitting colony formers. Similarly, BSP8, a macrocolony former, clearly enhanced the number of macrocolony and microcolony formers. When *M. t.* was added to the jar, the increase in population numbers was even more generalized: total bacteria, pectinolytic bacteria, and total number of fungi increased. Among the fungi, *M. t.* generally remained dominant. The flush of *M. t.* and BSP8 populations observed should largely be seen as corresponding not only with introduction of BSP8 and *M. t.* into the system, but also with their decomposing activity on *M. spicatum* and the nutrient release that stimulated the various population successions observed. Thus, a measure of the effectiveness of BSP8 and *M. t.* to bring about *M. spicatum* decline is their general enhancement of the number of other associated microorganisms.

134. Visual evaluations were used to confirm the multiple effects achieved by *M. t.* with its cellulolytic ability and BSP8 with its strong pectinolytic ability and capacity to exercise a hormonelike stress on *M. spicatum*. This latter effect was manifested in significant internodal elongation and appeared to predispose the plant to subsequent assault by *M. t.* and ultimate decomposition. In initial studies the organisms, prior to application, had accordingly been grown on cellulase-inducing medium or induced for pectinase production. However, due to the lengthy incubation required for growth and induction of enzymes (14 days for *M. t.* culture and 5 days for BSP8 culture), a change was made to media that would maximize cell yield in a minimum time. This was dictated by the increase in the volume of inoculum required for field studies. Fermentation techniques were adopted which involved enriched media with the elimination of the enzyme-induction process. This reduced incubation time to 72 hr for *M. t.* and 48 hr for BSP8. To test whether *M. t.* and BSP8 would continue to be active against *M. spicatum*, jar-scale tests were performed with organisms grown under the revised protocol.

135. The difference in inoculum procedures resulted in changes in the pattern of *M. spicatum* decline after exposure to the control agents. Previous plant decline associated with the application of *M. t.* (Gunner 1983) had been marked by the appearance of necrotic areas. In the results achieved by the application of cultures grown in the new media, symptoms of systemic plant decline appeared: the browning of plant tissue, the collapse of stems, and the ultimate disintegration of the plants. It is noteworthy, however, that BSP8 continued to produce a hormonelike response in *M. spicatum*.

136. The most rapid response to the organisms grown under the new protocol was observed in the laboratory with plant tips maintained in jars. Although effects identical to previous results were obtained with BSP8 grown under the new regimen, *M. t.* (with or without the bacterium) exercised an enhanced ability to bring about plant decline, where previously the action of BSP8 had been required. It may be speculated that the enhanced impact derives from the addition of vitamins and mineral salts to the growth medium. That the most rapid and dramatic results were obtained in the jar-scale experiments can perhaps be attributed to the sustained high concentration of the inoculum and the isolation of the plants from other environmental pressures.

137. Striking confirmation of these results was provided by the SEM. Uninoculated plant tissues revealed a generally sparse population of the native flora on the surfaces. This was dramatically altered by inoculation with BSP8 and *M. t.* Not only was there a profusion of microbial cells on the plant surface of various types, but the *M. t.* is also seen to proliferate and actually penetrate the host tissue. The decomposition that followed hastened the release of nutrients and the increase in total populations consistently observed.

138. In the pool experiments, the most significant result obtained was the restriction in biomass after treatment with *M. t.* either alone or in combination with BSP8. These data were further validated by the appearance of the treated plants after their removal from the pools: greater tissue decay, separation of stem from root, and scarcity of new tips. In a sense this may be seen as the acceleration of the cycle of growth and decline. From an ecosystems point of view, this weakening of *M. spicatum* could be seen as a portal for the reentry of competing plant populations in a natural environment.

139. In the pool setting, the application of BSP8 alone again resulted in increased internodal length. There was also a slight delay in the growth

cycle, as evidenced by the appearance of the plant and the number of growing tips. However, there was no significant difference in biomass between untreated controls and bacteria-treated pools. Thus, bacterial action must be considered to be capable of enhancing *M. t.* action, but not by itself exercising a lethal effect. This is supported by the biomass data in which joint *M. t.*-BSP8 treatment resulted in the greatest decline in biomass. However, the limitations of the pool environment in replicating the natural setting must not be overlooked. The shallow depth of the pool made more drastic the effects of temperature change and light penetration, among other variables (Barko, Adams, and Clesceri 1986). In particular, the intrusion of epiphytic algae obscured visual evaluation of the plant in situ, and more accurate representation of their condition could be obtained only when plants were removed from the pool and observed individually.

140. The dynamics of bacterial and fungal population numbers both in the pool and field settings (in 1985, 1986, and 1987) show a consistent relationship between communities in the water column and those associated with the plant. The populations are higher on the plant tissues, reflecting the richer nutritional base provided by the plant. The perturbation in the numbers of pectinolytic organisms could reflect variability in available substrate, the release of inhibitory and stimulatory factors from the plant, and predatory pressures. A further similarity is seen in the relatively smaller number of fungal propagules recovered from the water profile than from the plant surface. The fungal inoculum, as shown in the 1986 field experiment, was present in all readings from the plant surface, reflecting its affinity for the plant.

141. In the field setting of 1985, a clearly sustained rise in the microbial populations subsequent to the second inoculation was observed. This rise suggests that the plants, by releasing additional substrates or by having available more ecosites, generated enhanced colonization opportunities. This may reflect the effects of the first inoculation as well as seasonal changes in plant condition.

142. In addition to the phenomena described above, in 1986, the inoculum was distributed for the first time by a conventional air-pressure sprayer typically used for chemical applications. The pressure exerted at the nozzle discharge point was 200 psi (1,380 kPa) and, clearly, from the numbers of viable organisms recaptured at application time, there was significant

mortality due to shearing. The second application, through a manifold, at a pressure of ca. 5 psi (34 kPa) resulted in a two- to three-order of magnitude increase in viable cells at the time of inoculation. Ultimately, the first inoculum appeared to be below the infection threshold necessary, while the second proved adequate to initiate plant decline.

143. The inordinately high water level of Stockbridge Bowl in the summer of 1986 appeared to provoke some changes in microbial numbers found in the water profile without the oscillations observed in the previous season, though microbial populations on the plant surface were consistent with those previously observed. This may reflect the larger dilution effect in the water profile, while the plant populations remain site-bound and nutrient-determined.

144. Significantly, peak levels of microbial populations (total, fungal, and pectinolytic) coincided with an increase in the index of plant decline obtained by visual evaluation of the plants in situ. One may speculate that the coincidence of increased numbers and the visual evidence of plant decline encompasses a number of events, which include the following: (a) the pectinolytic organisms may be seen as primary decomposers releasing nutrients by attacking plant components that may also provide additional ecosites for microbial populations, and (b) bacterial attachment generates plant stress, which serves as a predisposing element for successful fungal assault on the plant.

145. The results of the 1985 field study described the decline of the treated plants by visual criteria, but no significant difference in biomass between treated and control was obtained. This can be attributed to the difficulties in sequestering decayed plant material within the designated plots and preventing the intrusion of new growth from outside the treated area during the course of the experiment. In 1986, the biomass differences were dramatic. This would seem to reflect not only the lethal potential of the inoculum but also the improved methodology and the effective sequestering of the respective test sites. In this instance, there was very little possibility that an untreated plant could drift into the treated site and confound the biomass determination. The 25-fold increase in test plot size also mitigated against the intrusion into the treated site by new shoots generated from adjacent root systems. In addition, it appeared that the dieback generated by the treatment was much less readily obscured by the regeneration of tissue at the margins of the larger site.

146. The 10-fold reduction in root biomass observed may, in fact, be less than actually achieved since residual roots had to be pulled up from the sediment by scuba diving. Regardless of the limits of this harvesting technique, the effective decline in root tissue was evident. With respect to decline of stem-leaf tissue, the loss due to microbial intervention was no less apparent. The ratio of stem-leaf to root tissue for untreated plants was almost twice that of the *M. t.*-treated system. The high level of significance in the overall decline of plant, whether measured by residual stem-leaf or by root tissues, as a consequence of treatment by the microbial regimen employed confirmed that this technique is an effective agent for the control of Eurasian watermilfoil.

147. It is important to note that the applications methodology in 1987 differed substantially from that employed previously. The original procedure included the inoculation of a strongly pectinolytic and growth-stressing bacterium together with *M. t.* Laboratory trials, however, had indicated that *M. t.* alone could be effective in bringing about plant decline. In the interests of simplifying the application, as well as reducing fermentation costs, it was decided that the 1987 field trials would be conducted with *M. t.* alone.

148. The results indicated that although a significant level of control was achieved in the absence of the companion bacterium, there were qualitative differences in the results. While in 1986 virtual disappearance of plant material was noted in the treated site, in 1987 there appeared to be a random deletion in growth.

149. A number of elements that may have functioned to differentiate the 1987 results from previous applications include the time of application and the number of applications. As our results have indicated, the efficacy of *M. t.* is both temperature and dose conditioned, with maximum results achieved at ca. 25° C and with increasing dose levels. The 1987 application was made 1 month later than in 1986, at cooler ambient temperatures. Too, in distinction to the 1986 field study, in which two applications of both bacterium and fungus were made, in 1987 only one application of the fungus was provided. Nonetheless, a strong measure of control is indicated even in these circumstances. It should be emphasized, however, that maximum control potential rests with applications made at the highest summer temperatures and with the maximal dose practicable.

150. Consonant with our previous studies, population dynamics reflect the strong microfloral commitment to the plant host. Numbers of organisms in the water profile are invariably several orders of magnitude lower than those on plant surfaces, reflecting diminished substrate possibilities. However, on the plant, where secretions, sloughed off tissue components, and saprophytic possibilities exist, the higher numbers reflect these substrate options. It is of interest that populations differ with the different plant zones. While the roots serve as the richest substrate provider for bacteria, fungi tend to remain at similar densities on all areas of the plant.

151. The introduction of *M. t.* as inoculum, on the other hand, while raising its numbers on tips and the midsection, did not increase its presence on the roots. This may reflect the manner of *M. t.* application, just below the water surface, and accordingly the longer period of time required for the fungus to reach and establish itself on the plant root, in contrast to its rapid establishment on tips and stems.

152. In general, there was little perturbation of heterotrophic microflora in the phyllosphere while, in the rhizosphere, shifts were more pronounced. Most noticeable was the change in the pectinolytic, pitting microflora whose numbers increased significantly in the plant midsection subsequent to inoculation with *M. t.* This would logically follow the invasive action that would make more substrate available to the pectinolytics and stimulate a flush in their growth. Viewed overall, however, the introduction of *M. t.* does not have a lasting effect on population dynamics and thus in no way stresses the ecosystem.

153. The specificity of *M. t.* infectivity was tested with several varieties of aquatic and terrestrial plants with little evidence of pathogenicity. Even plants with a record of susceptibility to *M. t.* infection showed *M. t.* to be a very weak pathogen to only two of the species of clover (*Trifolium* sp.) tested. Since an inordinately high inoculum rate and direct physical application by brush was necessary, it would suggest that such infection is not readily achieved. Thus, it would seem that the limited potential of *M. t.* as a plant pathogenic agent would not be a factor in preventing its application in an aquatic system.

PART V: CONCLUSIONS AND RECOMMENDATIONS

154. The validity of our thesis that biological control of a target species could be achieved by the manipulation of its associated microflora was confirmed in the instance of Eurasian watermilfoil, *Myriophyllum spicatum*.

155. Extension of our studies through jar, pool, and field experiments has provided a consistent demonstration that an explicit decline of *M. spicatum* could be achieved by the application of *Mycoleptodiscus terrestris* alone or with *Bacillus* sp. strain P8. These microorganisms were originally isolated from the plant and selected for their compatibility with each other, their capacity to compete with normal residents in the phyllosphere of Eurasian watermilfoil, their ability to resist the inhibitory action of phenolic compounds produced by the plant, and their capacity to degrade specific plant tissues. No significant residual increase in the numbers of the effective organisms was observed that would suggest perturbations in the aquatic ecosystem. The very low pathogenic potential of *M. terrestris* to a number of terrestrial plants previously identified as hosts to this fungus would not appear to restrict its use in an aquatic setting.

156. Control of *M. spicatum* can be achieved with *M. t.* alone, with appropriate regard for water temperature, inoculum density, and plant physiological status. To maximize plant kill, the time of application should be at the highest possible water temperature in the natural setting and the application process adjusted to ensure sufficient contact between plant and inoculum.

157. More extensive field trials among a range of settings that would embrace a variety of temperatures, lake conditions, and seasonal applications should be initiated.

158. Fermentation and formulation procedures should be optimized and application techniques developed to ensure maximum efficacy.

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Table 1

Effects of Various Densities of Microbial Populations from the Tip Area of *M. spicatum* Upon the Growth of *M. terrestris* on Selected Culture Media

Medium	Microorganisms Recovered cfu/ml	Inoculum Dilutions	Average Size of *M. t.* Colonies*	P-Value** 10^{-1}	10^{-3}	10^{-4}	10^{-5}
TSA	2.5×10^5	10^{-1}	0.55	1.00			
		10^{-3}	2.13	0.00††	1.00		
		10^{-4}	2.93	0.00††	0.00††	1.00	
		10^{-5}	2.60	0.00††	0.00††	0.04†	1.00
		Control	2.60	0.00††	0.00††	0.03†	1.00
PDA	9.1×10^3	10^{-1}	1.73	1.00			
		10^{-3}	2.55	0.00††	1.00		
		10^{-4}	2.68	0.00††	0.46	1.00	
		10^{-5}	2.63	0.00††	0.69	0.78	1.00
		Control	3.00	0.00††	0.03†	0.08	0.08
PA	1.87×10^5	10^{-1}	0.60	1.0			
		10^{-3}	2.23	0.00††	1.00		
		10^{-4}	2.90	0.00††	0.00††	1.00	
		10^{-5}	3.35	0.00††	0.00††	0.04†	1.00
		Control	3.00	0.00††	0.00††	0.45	0.08
MA	9.1×10^2	10^{-1}	3.13	1.00			
		10^{-3}	3.45	0.04†	1.00		
		10^{-4}	3.68	0.00††	0.03†	1.00	
		10^{-5}	3.98	0.00††	0.00††	0.02†	1.00
		Control	3.78	0.00††	0.02†	0.30	0.11

* Average size obtained by averaging four sets of replicates. Each replicate was measured twice for shortest and longest diameter and averaged before final calculation.

** P-values obtained from analysis of variance F values and t-test of four sets of replicates.

† $P \leq 0.05$, significant.

†† $P \leq 0.01$, highly significant.

Table 2

Effects of Various Densities of Microbial Populations from the Stem Area of *M. spicatum* Upon the Growth of *M. t.* on Selected Culture Media

Medium	Microorganisms Recovered cfu/ml	Inoculum Dilutions	Average Size of *M. t.* Colonies*	P-Value** 10^{-1}	10^{-3}	10^{-4}	10^{-5}
TSA	2.84×10^5	10^{-1}	0.60	1.00			
		10^{-3}	2.18	0.00††	1.00		
		10^{-4}	2.80	0.00††	0.00††	1.00	
		10^{-5}	2.63	0.00††	0.02†	0.25	1.00
		Control	2.60	0.00††	0.02†	0.17	0.86
PDA	1.2×10^3	10^{-1}	1.10	1.00			
		10^{-3}	2.68	0.00††	1.00		
		10^{-4}	2.53	0.00††	0.38	1.00	
		10^{-5}	2.53	0.00††	0.42	1.00	1.00
		Control	3.00	0.00††	0.09	0.03†	0.04†
PA	2.89×10^5	10^{-1}	0.55	1.0			
		10^{-3}	1.90	0.00††	1.00		
		10^{-4}	2.65	0.00††	0.00††	1.00	
		10^{-5}	2.90	0.00††	0.00††	0.06	1.00
		Control	3.00	0.00††	0.00††	0.02†	0.42
MA	8.5×10^2	10^{-1}	3.23	1.00			
		10^{-3}	3.73	0.00††	1.00		
		10^{-4}	3.75	0.01††	0.84	1.00	
		10^{-5}	3.73	0.02†	1.00	0.88	1.00
		Control	3.78	0.00††	0.60	0.85	0.72

* Average size obtained by averaging four sets of replicates. Each replicate was measured twice for shortest and longest diameter and averaged before final calculation.

** P-values obtained from analysis of variance F values and t-test of four sets of replicates.

† $P \leq 0.05$, significant

†† $P \leq 0.01$, highly significant.

Table 3

Effects of Various Densities of Microbial Populations from the Root Area of *M. spicatum* Upon the Growth of *M. t.* on Selected Culture Media

Medium	Microorganisms Recovered cfu/ml	Inoculum Dilutions	Average Size of *M. t.* colonies*	P-Value** 10^{-1}	10^{-3}	10^{-4}	10^{-5}
TSA	2.89×10^5	10^{-1}	0.55	1.00			
		10^{-3}	2.33	0.00††	1.00		
		10^{-4}	2.50	0.00††	0.19	1.00	
		10^{-5}	2.70	0.00††	0.02†	0.13	1.00
		Control	2.60	0.00††	0.07	0.45	0.86
PDA	1.19×10^5	10^{-1}	1.13	1.00			
		10^{-3}	2.38	0.00††	1.00		
		10^{-4}	2.63	0.00††	0.20	1.00	
		10^{-5}	2.53	0.00††	0.50	0.66	1.00
		Control	3.00	0.00††	0.01††	0.08	0.04†
PA	2.1×10^5	10^{-1}	0.55	1.0			
		10^{-3}	2.15	0.00††	1.00		
		10^{-4}	2.73	0.00††	0.00††	1.00	
		10^{-5}	2.80	0.00††	0.00††	0.36	1.00
		Control	3.00	0.00††	0.00††	0.03†	0.09
MA	1.1×10^3	10^{-1}	2.95	1.00			
		10^{-3}	3.84	0.00††	1.00		
		10^{-4}	3.81	0.00††	0.71	1.00	
		10^{-5}	3.88	0.00††	0.68	0.54	1.00
		Control	3.78	0.00††	0.62	0.70	0.34

* Average size obtained by averaging four sets of replicates. Each replicate was measured twice for shortest and longest diameter and averaged before final calculation.

** P-values obtained from analysis of variance F values and t-test of four sets of replicates.

† $P \leq 0.05$, significant

†† $P \leq 0.01$, highly significant.

Table 4

Growth of *M. terrestris* in the Presence of Pectinolytic Bacteria

Source of *M. t.* Inoculum	Diameter, cm, of *M. t.* in the Presence of Pectinolytic Bacterial Isolate			
	BHP	MS11	P8*	YC3
Center of colony	0.80	2.78	0.63	0.65
2 mm from center	1.00	3.33	0.73	0.43
Perimeter of colony	1.65	2.93	1.25	1.08
1 mm outside of visible colony	0.00	0.00	0.00	0.00

* Isolate P8 = *Bacillus* sp. isolate P8 (BSP8).

Table 5

Growth Inhibition of Pectinolytic Bacteria Associated with *M. terrestris*

Source of *M. t.* Inoculum	Inhibition Zone, cm, of Pectinolytic Bacterial Isolate			
	BHP	MS11	P8*	YC3
Center of colony	1.40	2.28	0.00	1.05
2 mm from center	1.50	2.33	0.00	1.05
Perimeter of colony	1.13	2.13	0.00	0.73
1 mm outside of visible colony	0.00	1.05	0.00	0.00

* Isolate P8 = *Bacillus* sp. isolate P8 (BSP8).

Table 6

Differential Response to Phenolic Compounds Exhibited by Microflora Associated with *M. spicatum* after 24 hr on Sensitivity Plates (SP) and 5 Days in Broth Media (BM)

Phenolic Compound*	Conc. ppm	Bacterial Isolate** MS11 BM	MS11 SP	BSP BM	BSP SP	BHP BM	BHP SP	MH_2 BM	MH_2 SP	YC3 BM	YC3 SP
Ellagic acid	1,000	-	++	-	+++	-	+++				
	500	-	++	+	+++	+	+++				
	50	-	++	+	+++	+	+++				
Gallic acid	1,000	-	++	+	+	-	-				
	500	-	++	+	+++	-	-				
	50	+++	+++	++	+++	+	++				
Tannic acid	1,000	-	++	-	++	-	-			-	-
	500	-	++	+	+++	-	-			++	++
	50	+	+++	+	+++	+	++			+++	+++
3,5-Dimethoxy-4-Hydroxycinnamic acid	1,000	-	+	+	++	+	++				
	500	++	+++	+	+++	+	++				
	50	+++	+++	++	+++	+	+++				
Protocatechuic acid	1,000	-	++	+	++	-	-				
	500	-	++	++	+++	+	+++				
	50	+++	+++	++	+++	+	+++				
Shikimic acid	1,000	++	+++	++	++	+	+++				
	500	+++	+++	++	+++	+	+++				
	50	+++	+++	++	+++	+	+++				
Caffeic acid	1,000	-	+	-	-	-	-				
	500	-	++	++	+++	-	++				
	50	++	+++	++	+++	+	++				
Cinnamic acid	1,000	-	++	-	-	-	-			-	-
	500	++	+++	+	+++	+	++			++	+++
	50	+++	+++	+	+++	+	+++			+++	+++
m-Coumaric acid	1,000	-	++	-	++	+	-				
	500	++	+++	+	+++	+	++				
	50	+++	+++	++	+++	+	+++				

(Continued)

* From Planas et al. 1981.

** Symbols are defined as follows: -, no growth; +, scanty growth; ++, moderate growth; +++, abundant growth.

Table 6 (Concluded)

Phenolic Compound	Conc. ppm	Bacterial Isolate									
		MS11		BSP		BHP		MH_2		YC3	
		BM	SP	BM	SP	BM	SP	BM	SP	BM	SP
p-Coumaric acid	1,000	-	+	+	+	-	-				
	500	++	+++	+	++	+	++				
	50	+++	+++	++	+++	+	+++				
Ferrulic acid	1,000	++	+++	+	++	-	++				
	500	++	+++	++	+++	+	+++				
	50	+++	+++	++	+++	+	+++				
Gentisic acid (free)	1,000	+	++	+	+	+	+++				
	500	++	+++	+	+++	+	+++				
	50	+++	+++	+	+++	+	+++				
Gentisic acid (Na salt)	1,000	-	+++	+	+++	+	+++				
	500	++	+++	+	+++	+	+++				
	50	+++	+++	+	+++	+	+++				
Pyrogallol	1,000	-	++	-	++	-	-				
	500	-	+++	+	+	+	-				
	50	++	+++	+	+++	+	++				
Quinnic acid	1,000	++	++	+	+++	+	++				
	500	+++	+++	++	+++	+	+++				
	50	+++	+++	++	+++	+	+++				
Syringic acid	1,000	+	+++	+	-	-	-	+++	+++		
	500	++	+++	++	+++	+	+++	+++	+++		
	50	+++	+++	++	+++	+	+++				

Table 7

Biomass of *M. spicatum* in Pools Treated with *M. t.* and BSP8

Treatment	Biomass Mean Value* g Dry Weight/Tray	p-Value** Control	BSP8	*M. t.*	*M. t.* and BSP8
Control	2.5733	1.0000			
BSP8	2.3621	0.3787	1.0000		
M. t.	1.9429	0.0044†	0.0592	1.0000	
M. t. and BSP8	1.7358	0.0044†	0.0081†	0.2077	1.0000

* Mean value of biomass obtained by averaging three sets of replicates from each treatment. Values represent average weight of plants from 24 trays (8 trays/replicate/containing 36 plants each).

** p-values obtained from analysis of variance F values and t-test of three sets of replicates.

† $P \leq 0.01$, highly significant.

Table 8

Results of Host Range Specificity Tests of *M. terrestris* on Selected Aquatic and Terrestrial Plants

Common Name	Scientific Name	Visible Symptoms*
Aquatic plants		
Corkscrew eelgrass	*Vallisneria* sp.	-
Duckweed	*Lemna minor*	-
Elodea	*Elodea (Anacharis)*	-
Grass leaf	*Sagittaria* sp.	-
Terrestrial Plants		
Green bean	*Phaseolus vulgaris*	-
Pea	*Pisum sativum* L.	-
Vetch	*Vicia* sp.	-
Wheat	*Triticum sativum*	-

* Observations were classified as either "+" (symptoms of infection observed) or "-" (no visible symptoms of infection observed).

Table 9

Infection Specificity of *M. terrestris*

Host Plant*		P-Value**	
Common Name	Scientific Name	Seedling	Mature
Red clover	*Trifolium pratense* L.	0.0000††	0.6780
White clover	*T. repens* L.	0.0000††	0.6622
Soybean	*Glycine max* L. merr.	0.2090	1.0000
Oats	*Avena* sp.	0.9433	0.0152†
Bluegrass var. 'Baron'	*Poa pratensis* L.	0.0006††	1.0000
Bluegrass var. 'Kentucky'	*Poa pratensis* L.	0.3180	0.0044††
Bluegrass var. 'Merion'	*Poa pratensis* L.	1.0000	0.0000††
Alfalfa var. 'Iroquis'	*Medicago sativa* L.	0.0000††	1.0000
Alfalfa var. 'Vernell'	*Medicago sativa* L.	0.0000††	0.3977
Alfalfa var. 'Saranac'	*Medicago sativa* L.	0.0000††	0.4747

* Previously reported as a host plant of *M. t.*
** P-values obtained from univariate and multivariate analysis of variance of 10 sets of replicates.
† $P \leq 0.05$, significant.
†† $P \leq 0.01$, highly significant.

Table 10

Effect of Treatment* on Biomass of Eurasian Watermilfoil in Stockbridge Bowl, Massachusetts (1985)

	Mean Biomass** (Dry Weight Plant Material, g/0.5 m^2)		
Plant Part	Control	Treated	P-value†
Dead tissue	30.02	40.94	0.31
Live tissue	62.53	50.96	0.17
Combined	92.55	91.90	0.97

* *Mycoleptodiscus terrestris* and *Bacillus* sp. isolate P8 applied as a biological control.
** Average weight of dried plant materials from 8 samples/0.5 m^2 after 12 weeks.
† P-values obtained from analysis of variance F values and t-test of eight sets of replicates.

Table 11

Effect of Treatment* on Biomass of Eurasian Watermilfoil in Stockbridge Bowl, Massachusetts (1985)

Plant Part	Mean Biomass** (Dry Weight Plant Material, g/0.5 m^2) Control	Treated	P-value†
Stem-leaf	133.99	8.23	0.0003††
Root	5.02	0.48	0.0036
Combined	136.29‡	8.71	0.0020

* *Mycoleptodiscus terrestris* and *Bacillus* sp. isolate P8 applied as a biological control.

** Average weight of dried plant materials from 8 samples/0.25 m^2/plot after 10 weeks.

† P-values obtained from analysis of variance F values and t-test of eight sets of replicates.

†† $P \leq 0.01$, highly significant.

‡ Discrepancy in total of stem-leaf plus root is due to loss of root tissue in one control sample.

Table 12

Effect of Treatment* on Biomass of Eurasian Watermilfoil in Stockbridge Bowl, Massachusetts (1987)

Plant Part	Mean Biomass** (Dry Weight Plant Material, g/0.25 m^2) Control	Treated	P-value†
Stem-leaf	60.81	16.50	0.003††
Root	3.98	2.23	0.190
Combined	64.79	18.73	0.002††

* *Mycoleptodiscus terrestris* applied as a biological control.

** Average weight in grams of dried plant material (105° C, 72 hr) from eight 0.25 m^2 samples/plot after 4 weeks.

† P-values obtained from analysis of variance F values and t-test of eight sets of replicates.

†† $P \leq 0.01$, highly significant.

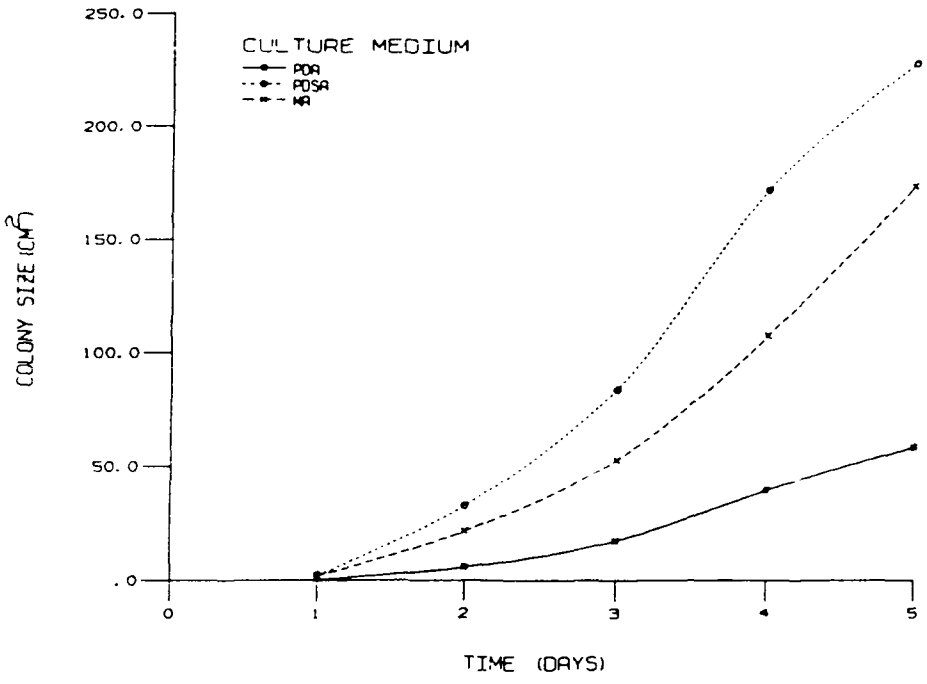

Figure 1. Growth curves of *M. terrestris* on PDA, PDSA, and MA media

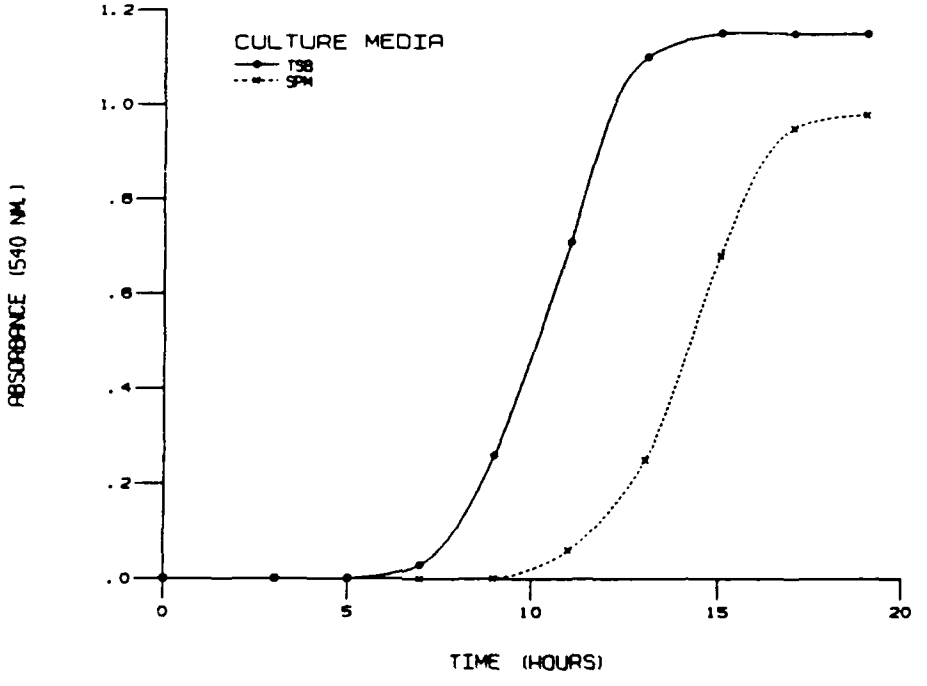

Figure 2. Growth curves of *Bacillus* sp. isolate P8 in TSB and SPM media

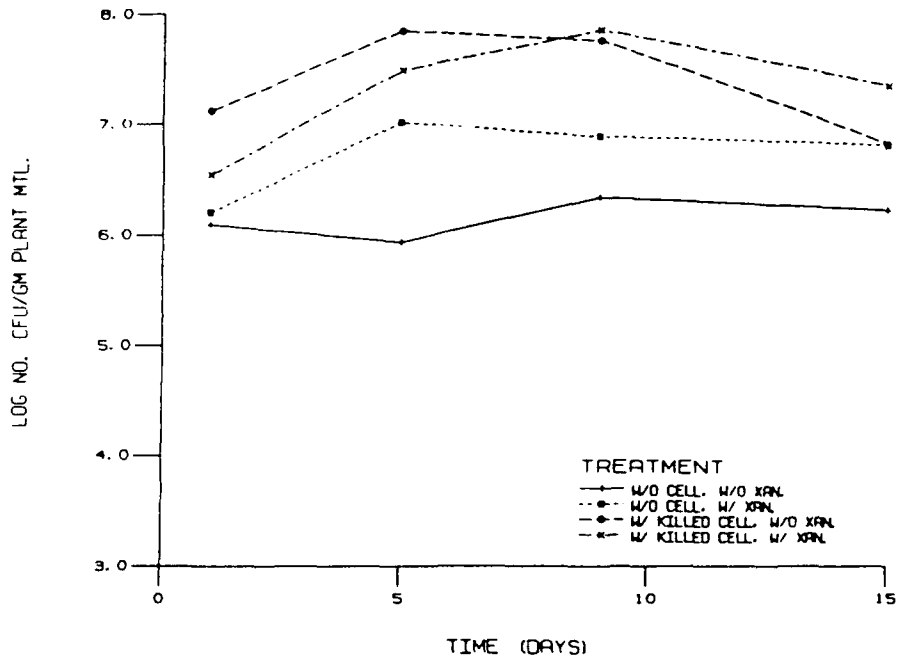

Figure 3. Bacterial populations recovered on TSA medium from plant tissues variously treated: CELL = *Bacillus* sp. isolate P8; XAN = 1% xanthan

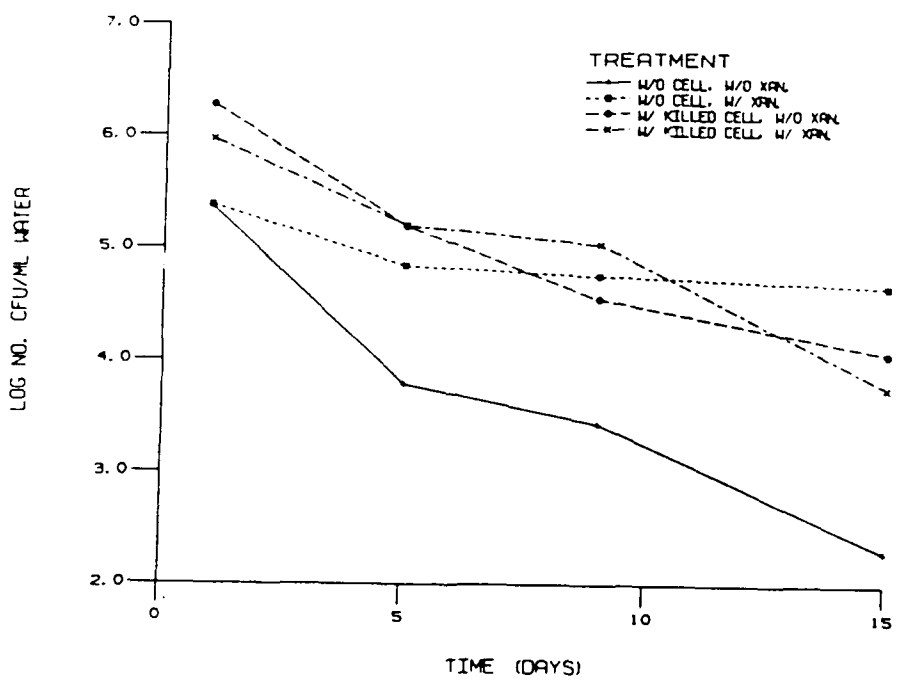

Figure 4. Bacterial populations recovered on TSA medium from water profiles variously treated: CELL = *Bacillus* sp. isolate P8; XAN = 1% xanthan

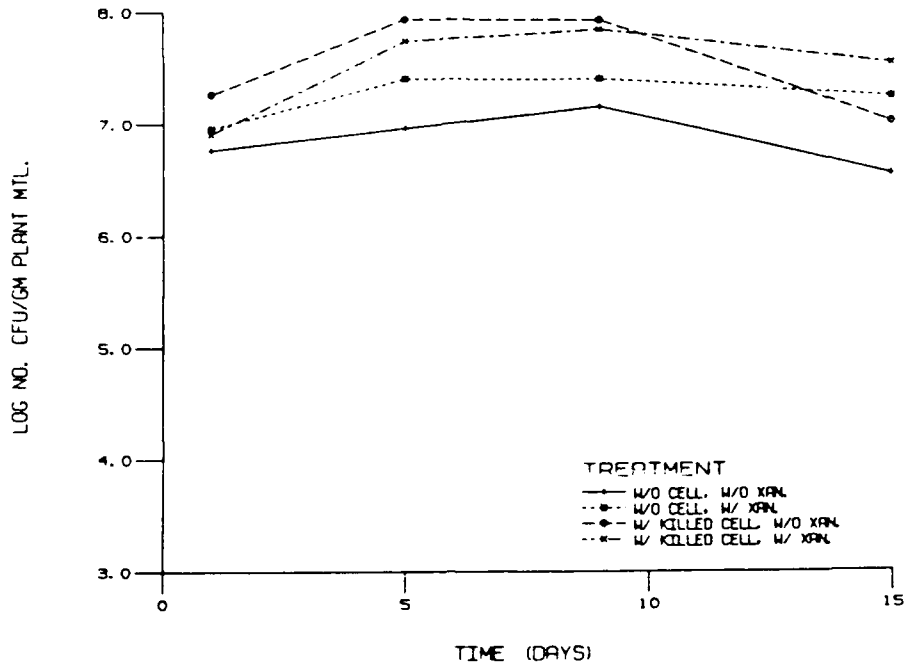

Figure 5. Pectinolytic bacterial populations recovered on PA medium from plant tissues variously treated: CELL = *Bacillus* sp. isolate P8; XAN = 1% xanthan

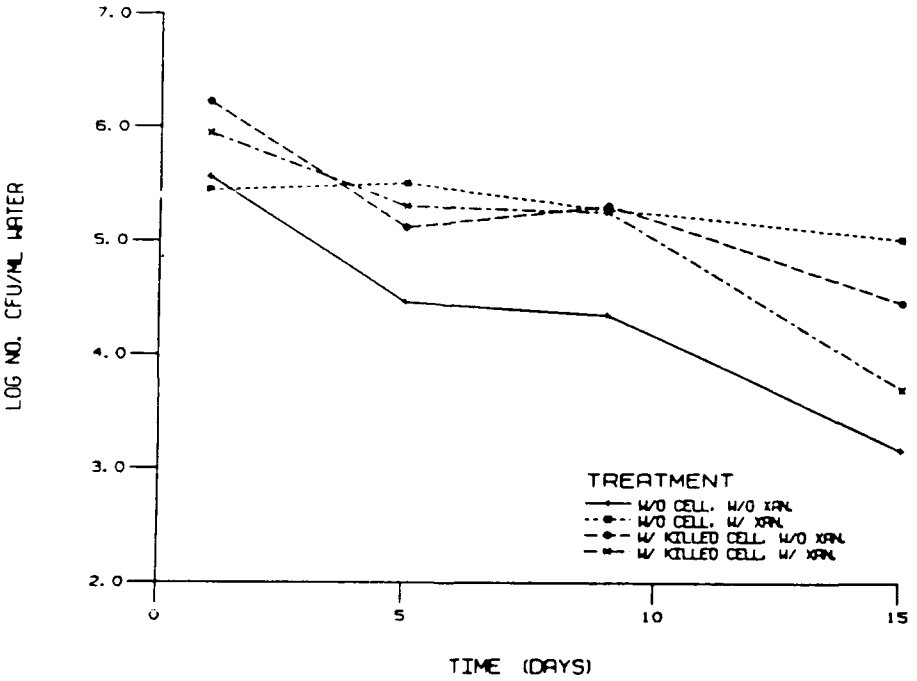

Figure 6. Pectinolytic bacterial populations recovered on PA medium from water profiles variously treated: CELL = *Bacillus* sp. isolate P8; XAN = 1% xanthan

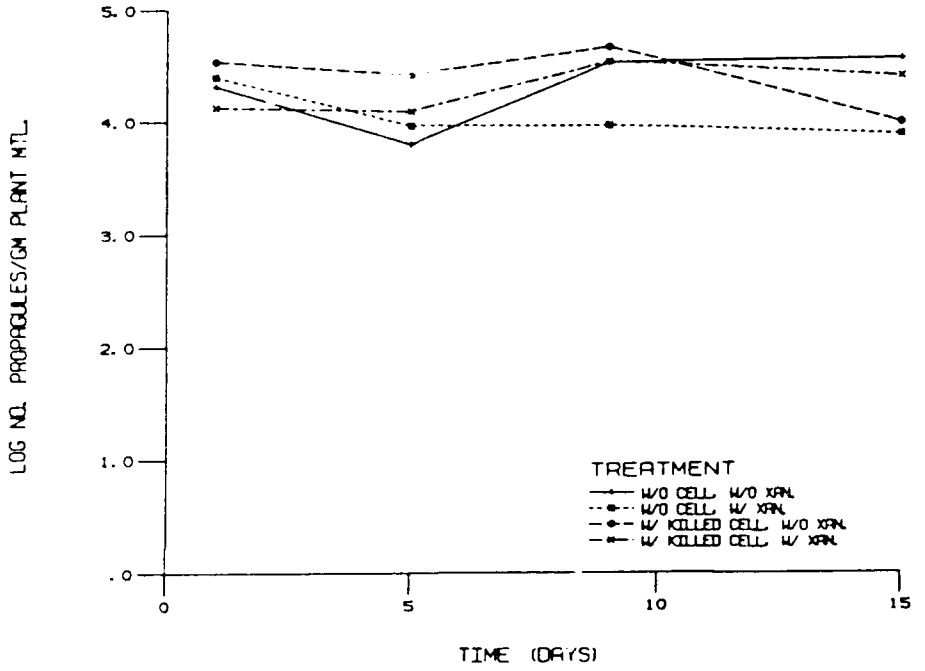

Figure 7. Fungal populations recovered on MA medium from plant tissues variously treated: CELL = *Bacillus* sp. isolate P8; XAN = 1% xanthan

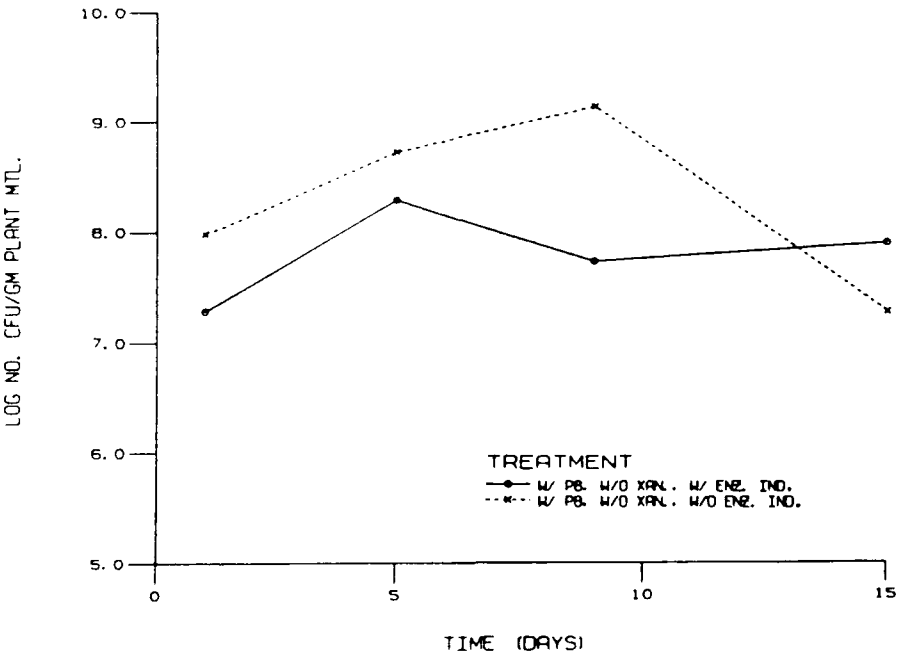

Figure 8. Bacterial populations recovered on TSA medium from plant tissues variously treated: P8 = *Bacillus* sp. isolate P8; XAN = 1% xanthan; ENZ. IND. = pectinolytic enzyme induction

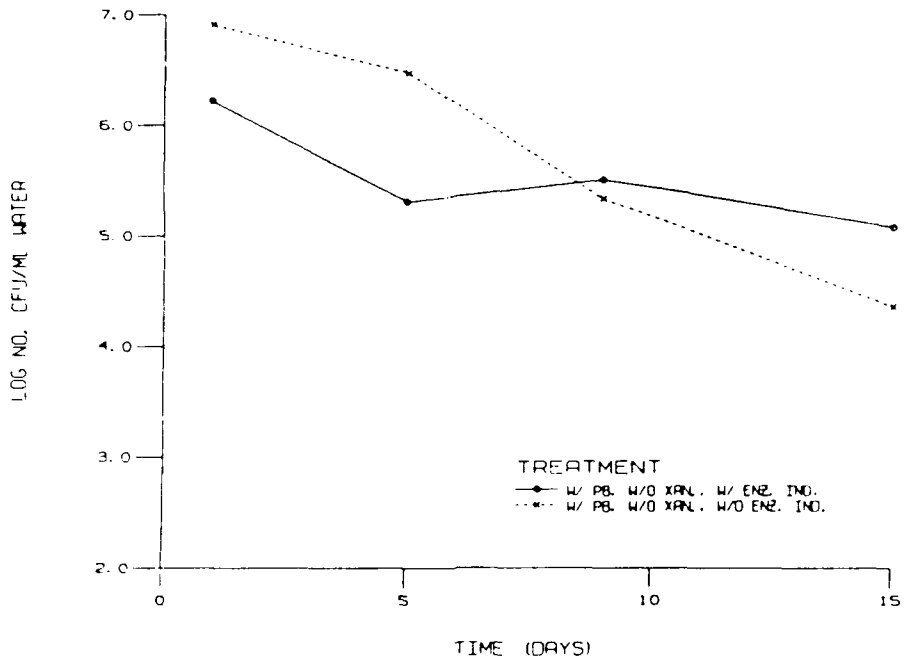

Figure 9. Bacterial populations recovered on TSA medium from water profiles variously treated: P8 = *Bacillus* sp. isolate P8; XAN = 1% xanthan; ENZ. IND. = pectinolytic enzyme induction

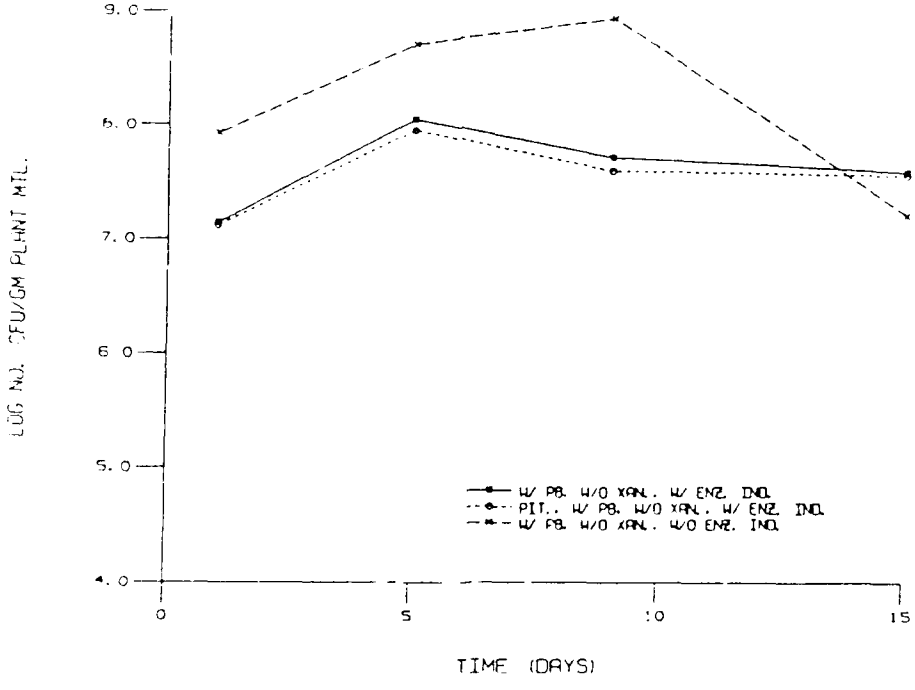

Figure 10. Pectinolytic bacterial populations recovered on PA medium from plant tissues variously treated: P8 = *Bacillus* sp. isolate P8; XAN = 1% xanthan; ENZ. IND. = pectinolytic enzyme induction; PIT = pitting colonies

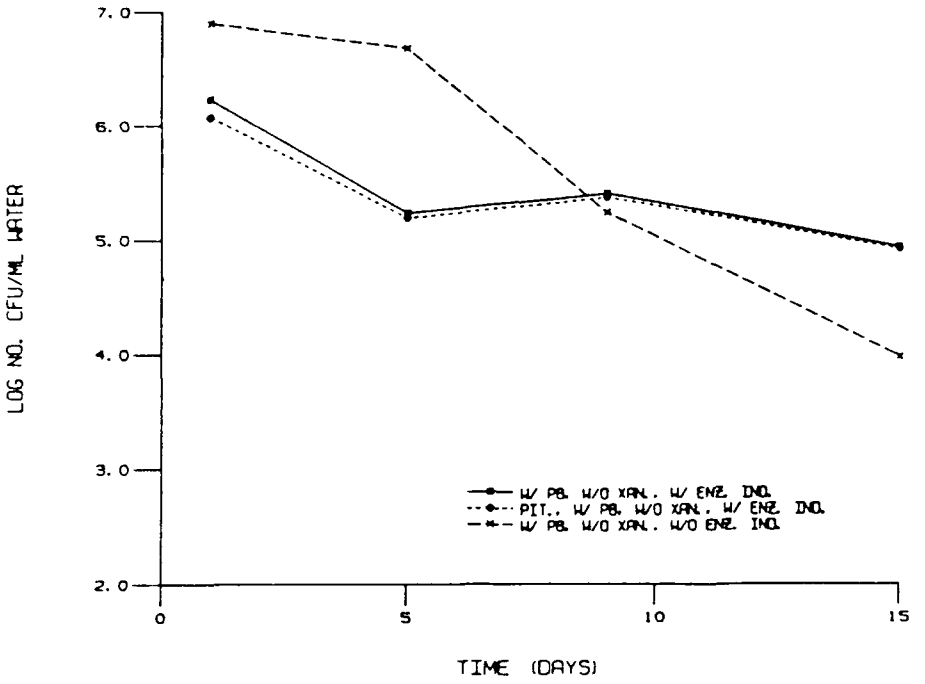

Figure 11. Pectinolytic bacterial populations recovered on PA medium from water profiles variously treated: P8 = *Bacillus* sp. isolate P8; XAN = 1% xanthan; ENZ. IND. = pectinolytic enzyme induction; PIT. = pitting colonies

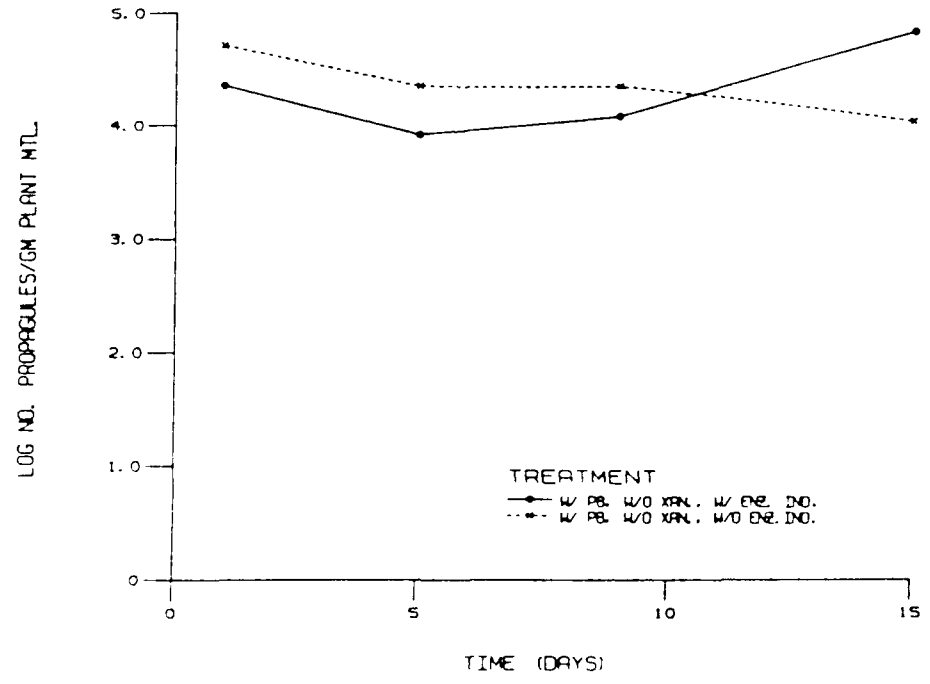

Figure 12. Fungal populations recovered on MA medium from plant tissues variously treated: P8 = *Bacillus* sp. isolate P8; XAN = 1% xanthan; ENZ. IND. = pectinolytic enzyme induction

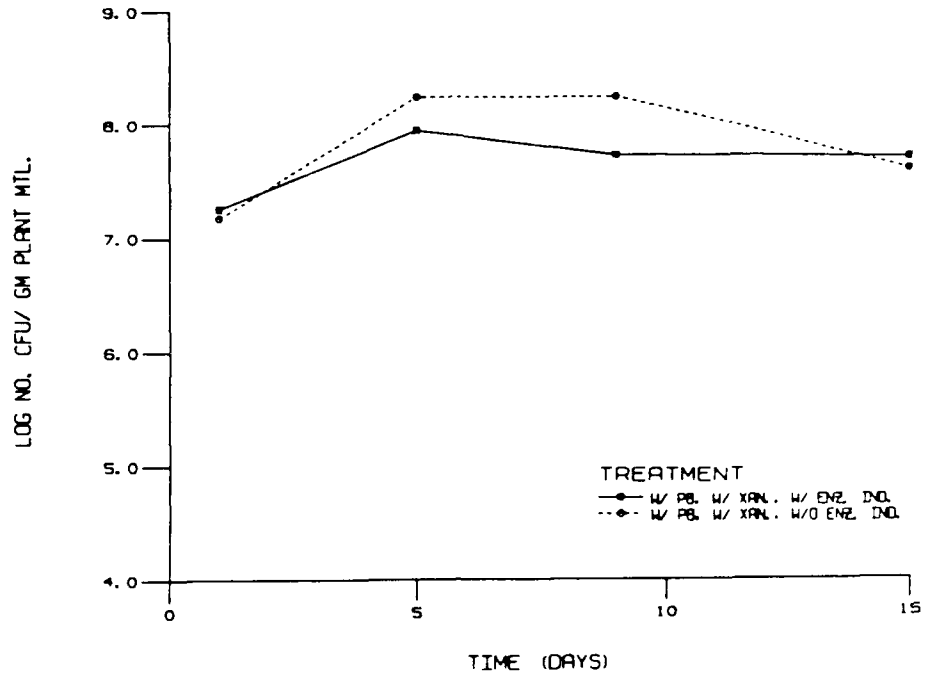

Figure 13. Bacterial populations recovered on TSA medium from plant tissues variously treated: P8 = *Bacillus* sp. isolate P8; XAN = 1% xanthan; ENZ. IND. = pectinolytic enzyme induction

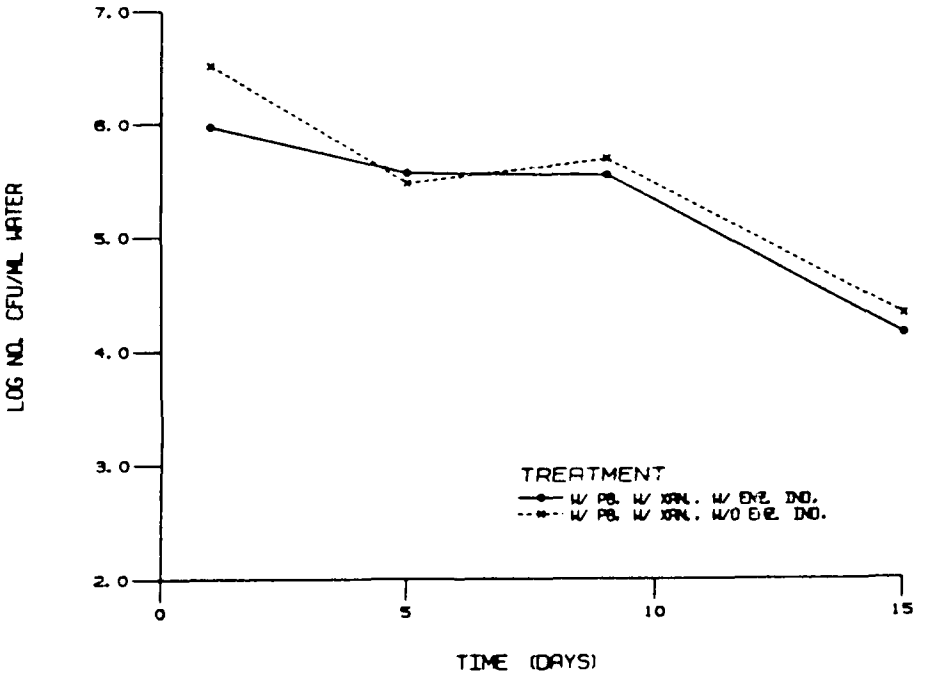

Figure 14. Bacterial populations recovered on TSA medium from water profiles variously treated: P8 = *Bacillus* sp. isolate P8; XAN = 1% xanthan; ENZ. IND. = pectinolytic enzyme induction

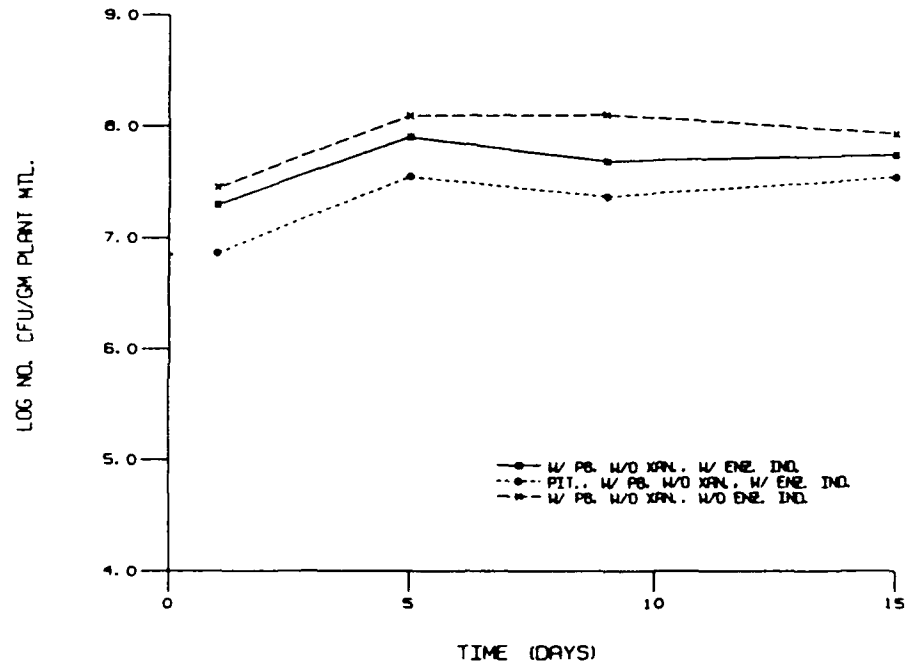

Figure 15. Pectinolytic bacterial populations recovered on PA medium from plant tissues variously treated: P8 = *Bacillus* sp. isolate P8; XAN = 1% xanthan; ENZ. IND. = pectinolytic enzyme induction; PIT. = pitting colonies

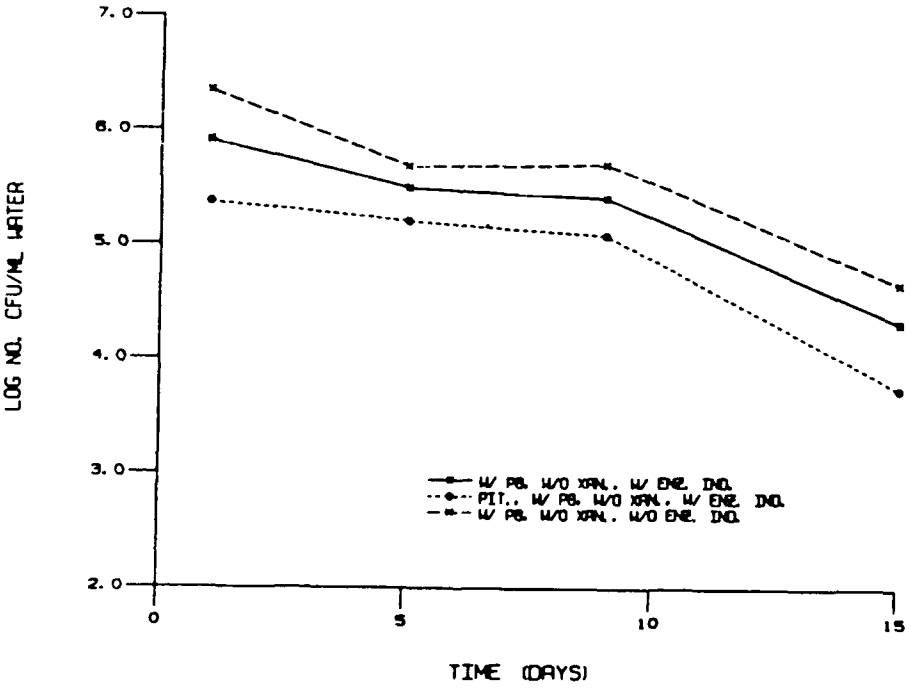

Figure 16. Pectinolytic bacterial populations recovered on PA medium from water profiles variously treated: P8 = *Bacillus* sp. isolate P8; XAN = 1% xanthan; ENZ. IND. = pectinolytic enzyme induction; PIT. = pitting colonies

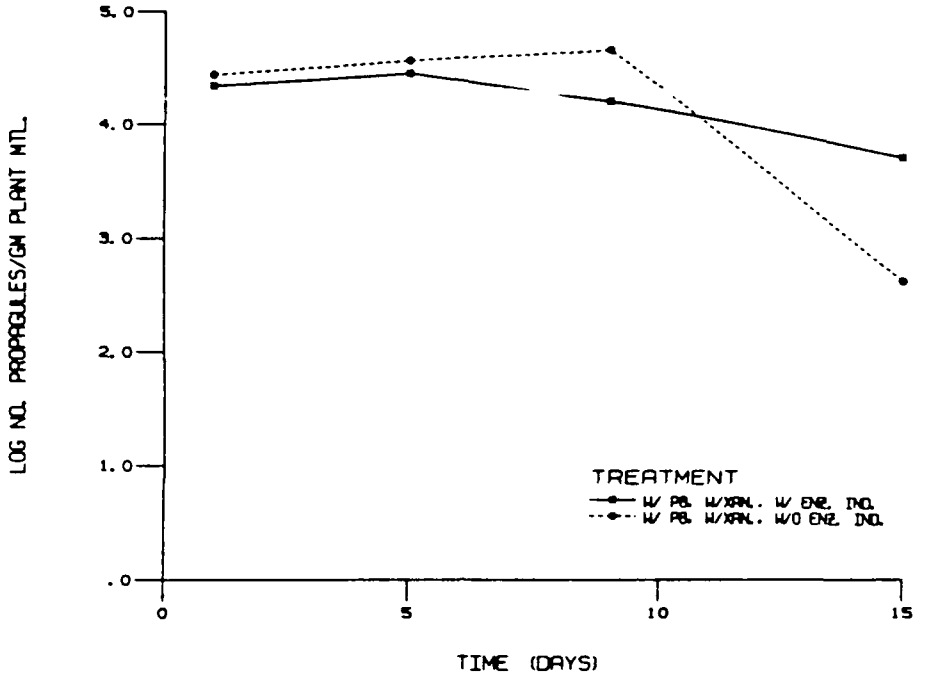

Figure 17. Fungal populations recovered on MA medium from plant tissues variously treated: P8 = *Bacillus* sp. isolate P8; XAN = 1% xanthan; ENZ. IND. = pectinolytic enzyme induction

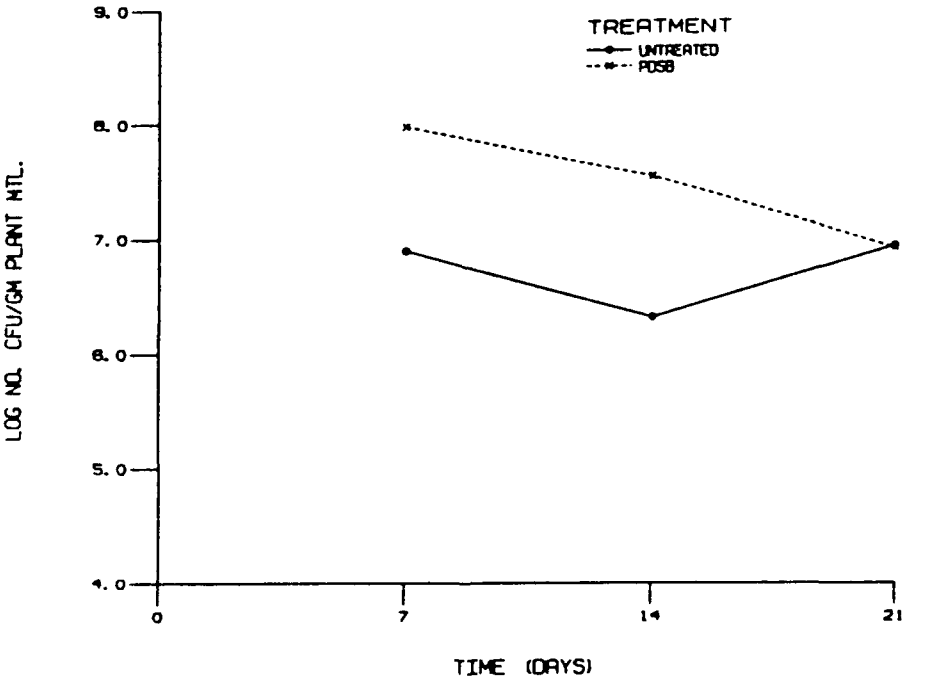

Figure 18. Bacterial populations recovered on TSA medium from untreated and PDSB-treated plant tissues

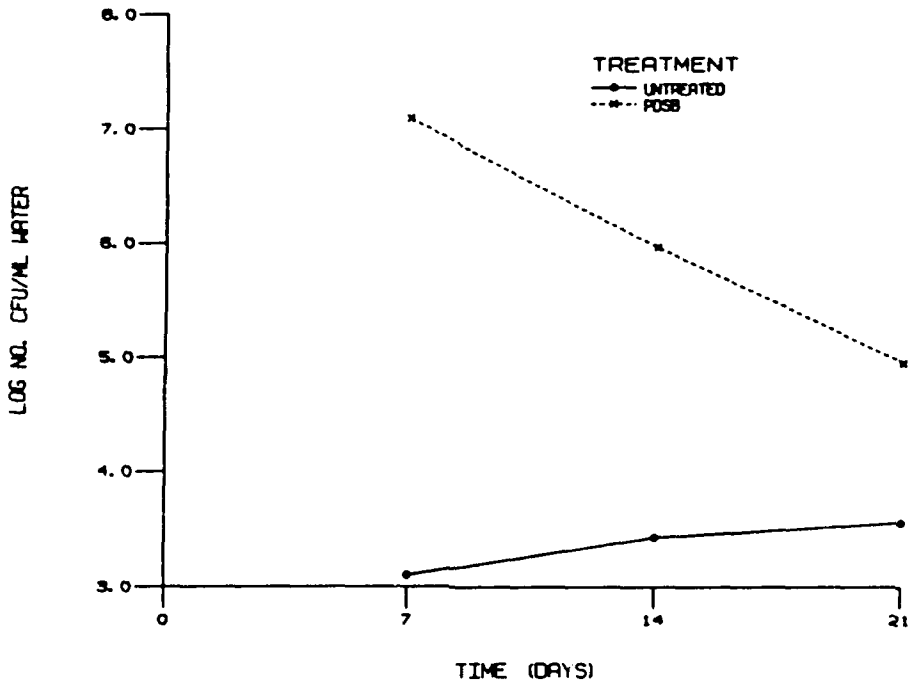

Figure 19. Bacterial populations recovered on TSA medium from untreated and PDSB-treated water profiles

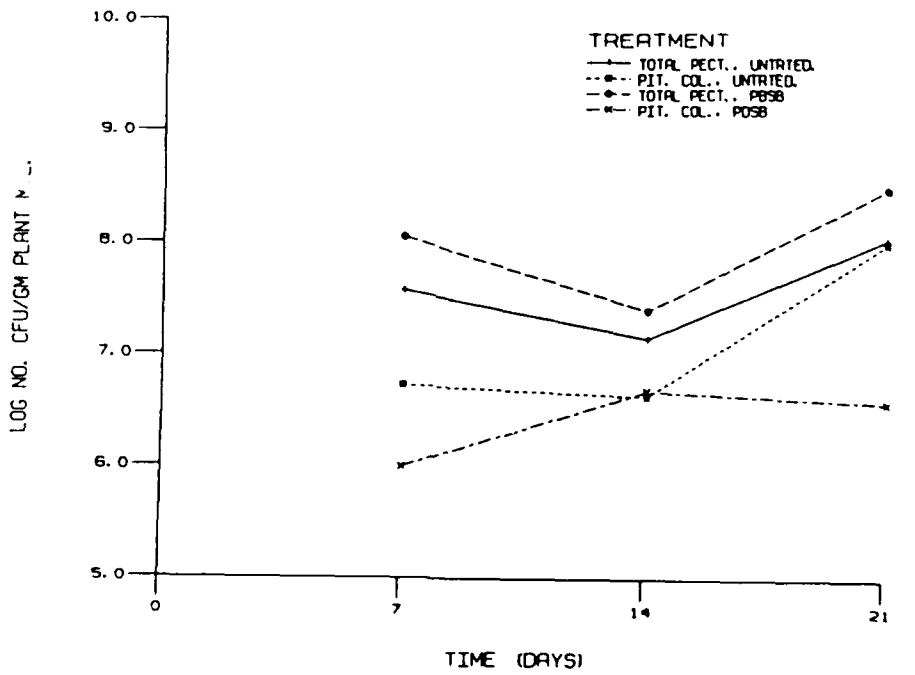

Figure 20. Total and strongly pectinolytic bacterial populations recovered on PA medium from untreated and PDSB-treated plant tissues

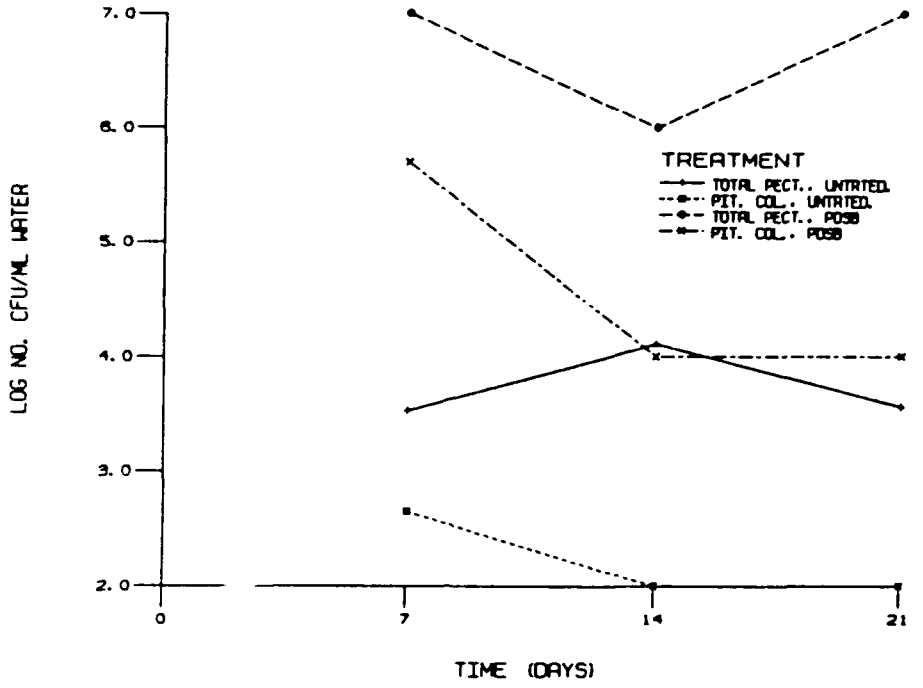

Figure 21. Total and strongly pectinolytic bacterial populations recovered on PA medium from untreated and PDSB-treated water profiles

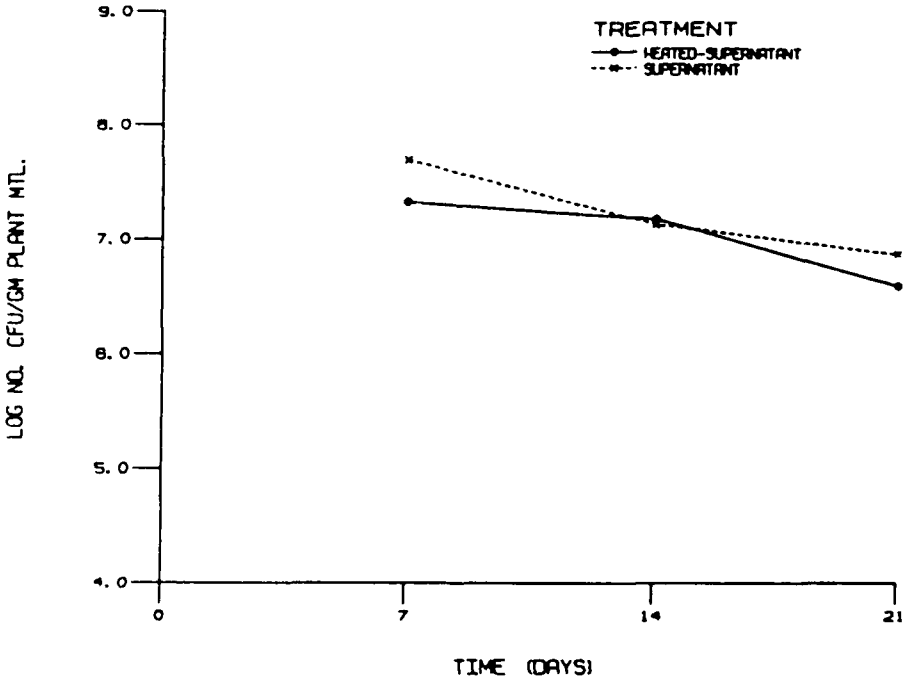

Figure 22. Bacterial populations recovered on TSA medium from plant tissues treated with heated and untreated supernatants

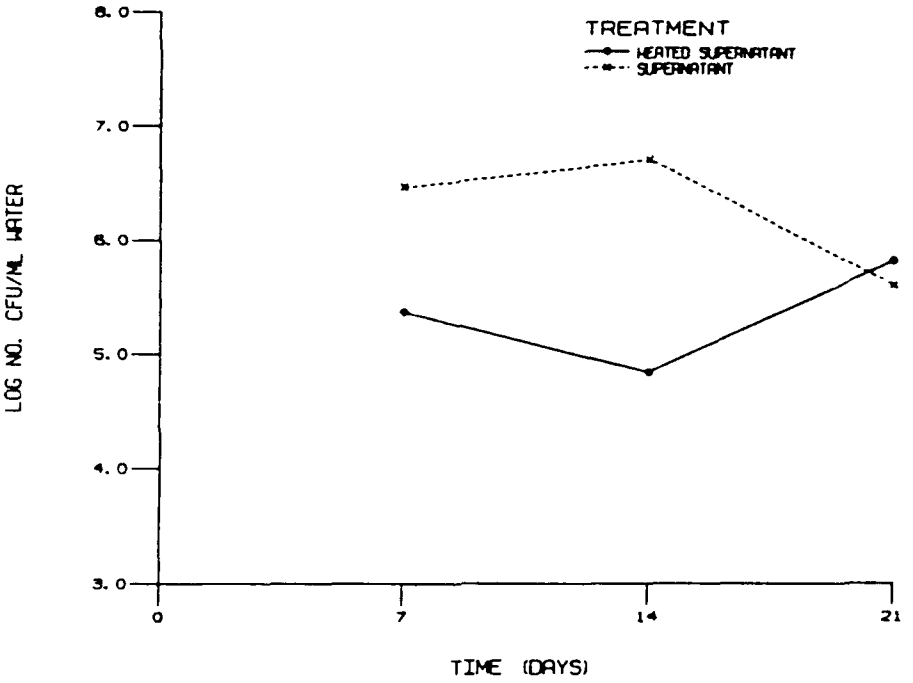

Figure 23. Bacterial populations recovered on TSA medium from water profiles treated with heated and unheated supernatants

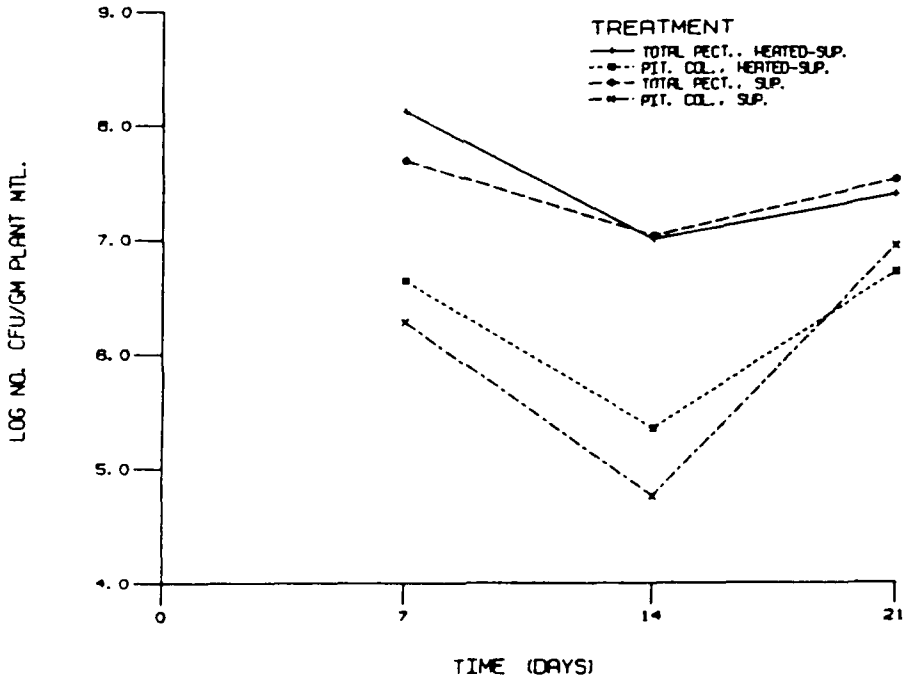

Figure 24. Total and strongly pectinolytic bacterial populations recovered on PA medium from plant tissues treated with heated and unheated supernatants

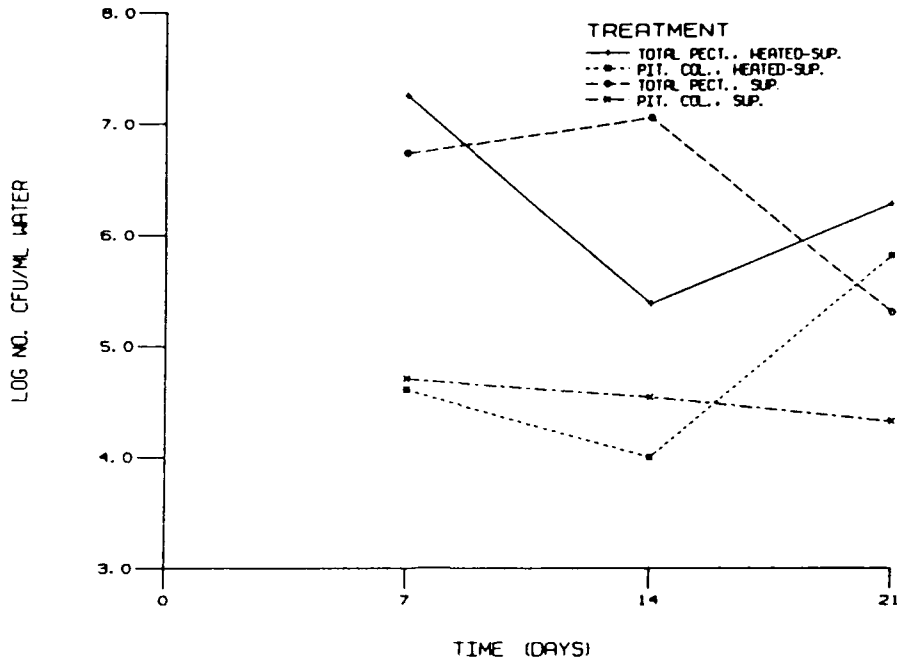

Figure 25. Total and strongly pectinolytic bacterial populations recovered on PA medium from water profiles treated with heated and unheated supernatants

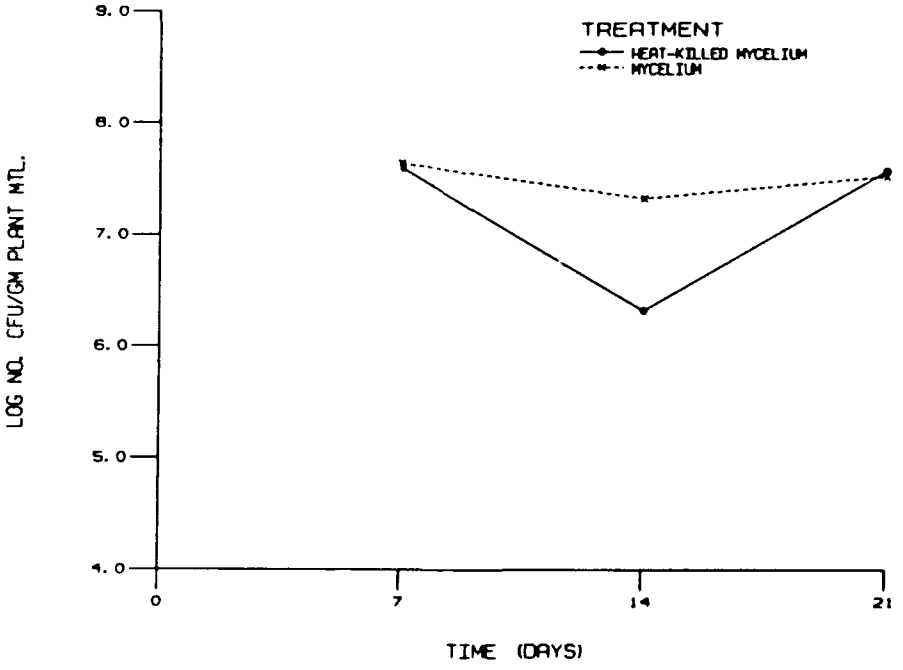

Figure 26. Bacterial populations recovered on TSA medium from plant tissues treated with heat-killed and live *M. t.* mycelia

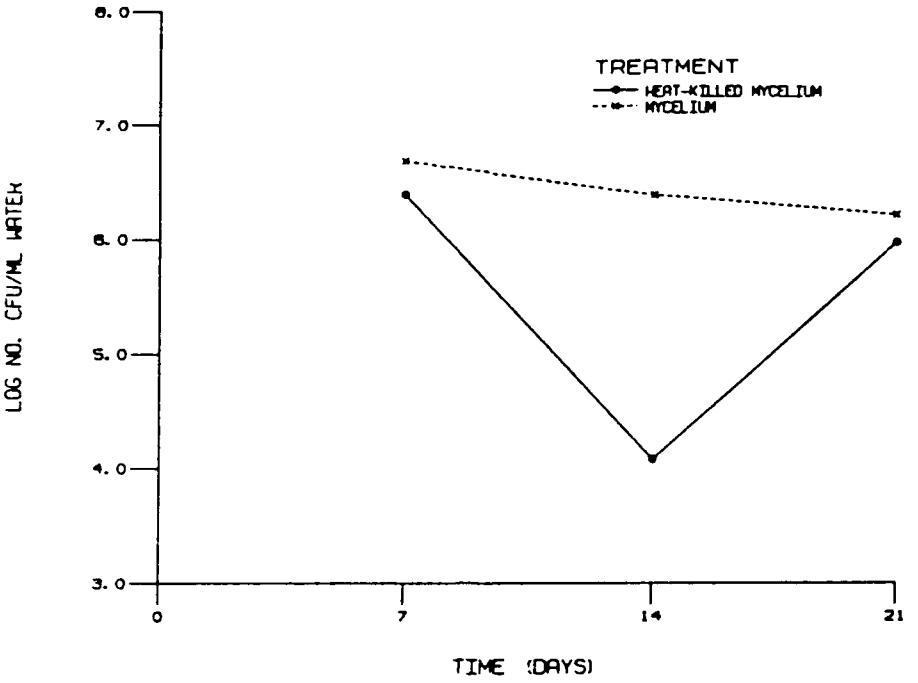

Figure 27. Bacterial populations recovered on TSA medium from water profiles treated with heat-killed and live *M. t.* mycelia

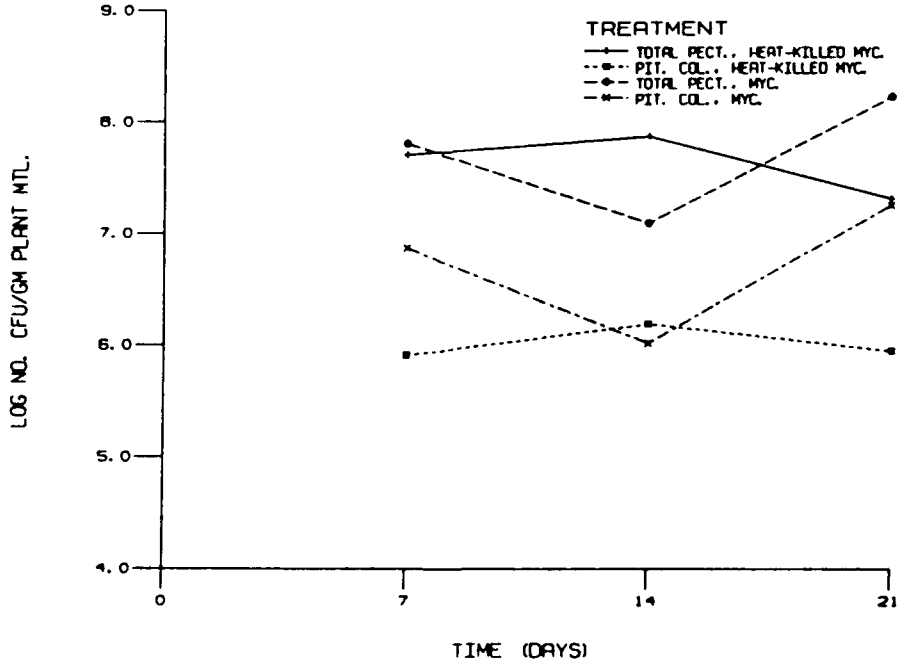

Figure 28. Total and strongly pectinolytic bacterial populations recovered on PA medium from plant tissues treated with heat-killed and live *M. t.* mycelia

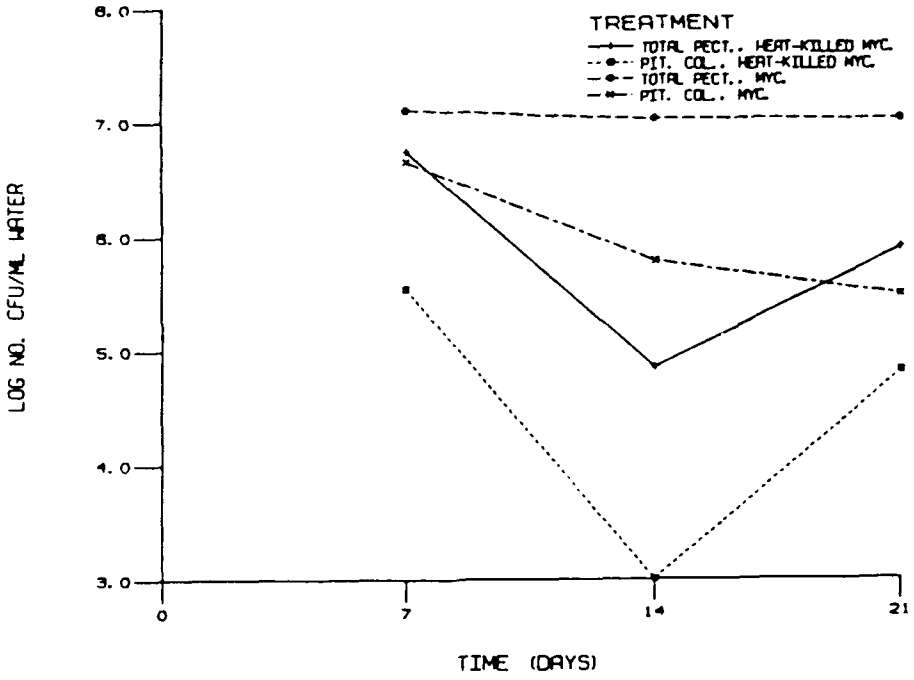

Figure 29. Total and strongly pectinolytic bacterial populations recovered on PA medium from water profiles treated with heat-killed and live *M. t.* mycelia

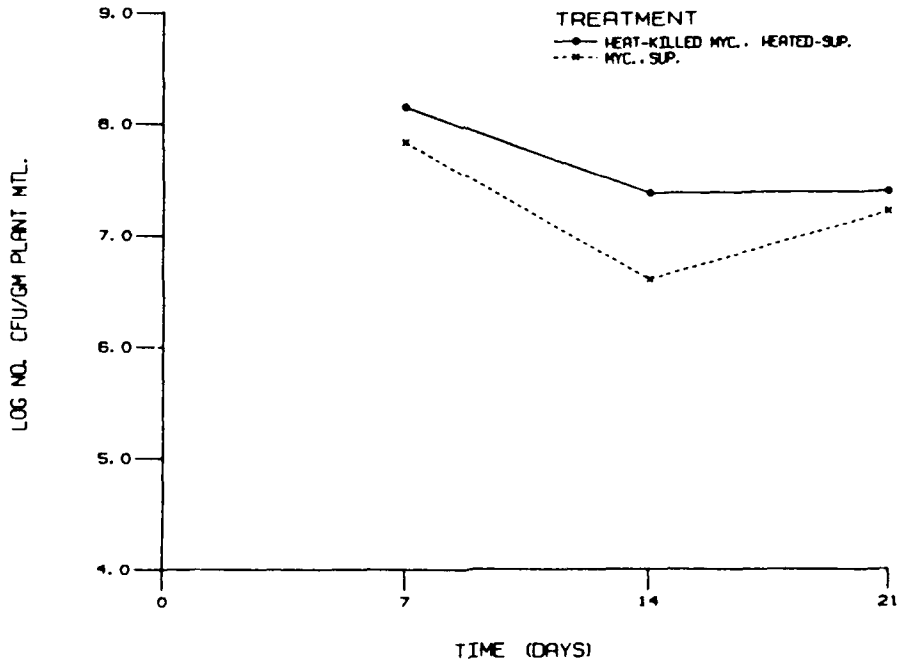

Figure 30. Bacterial populations recovered on TSA medium from plant tissues variously treated: MYC. = *M. t.* mycelia; SUP. = supernatant

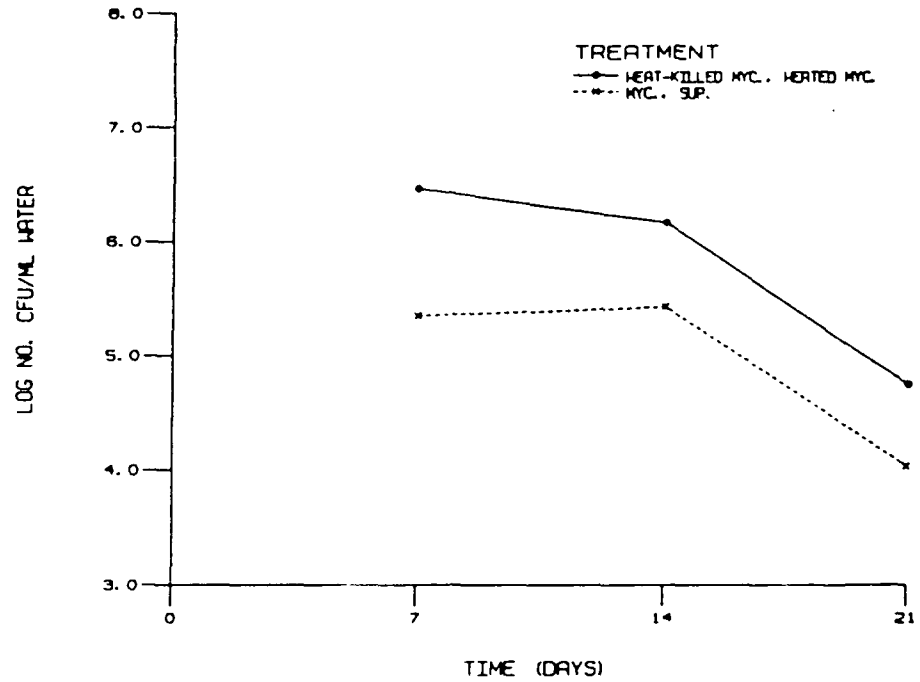

Figure 31. Bacterial populations recovered on TSA medium from water profiles variously treated: MYC. = *M. t.* mycelia; SUP. = supernatant

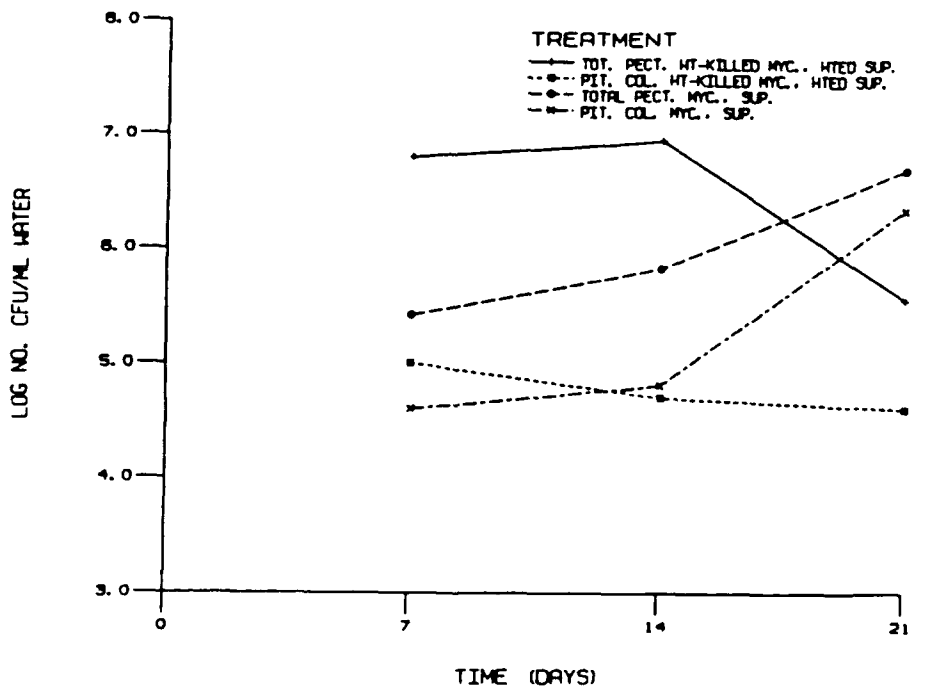

Figure 32. Total and strongly pectinolytic bacterial populations recovered on PA medium from water profiles variously treated: HT, HTED. = heat, heat-treated; MYC. = *M. t.* mycelia; SUP. = supernatant; PIT. COL. = pitting colonies

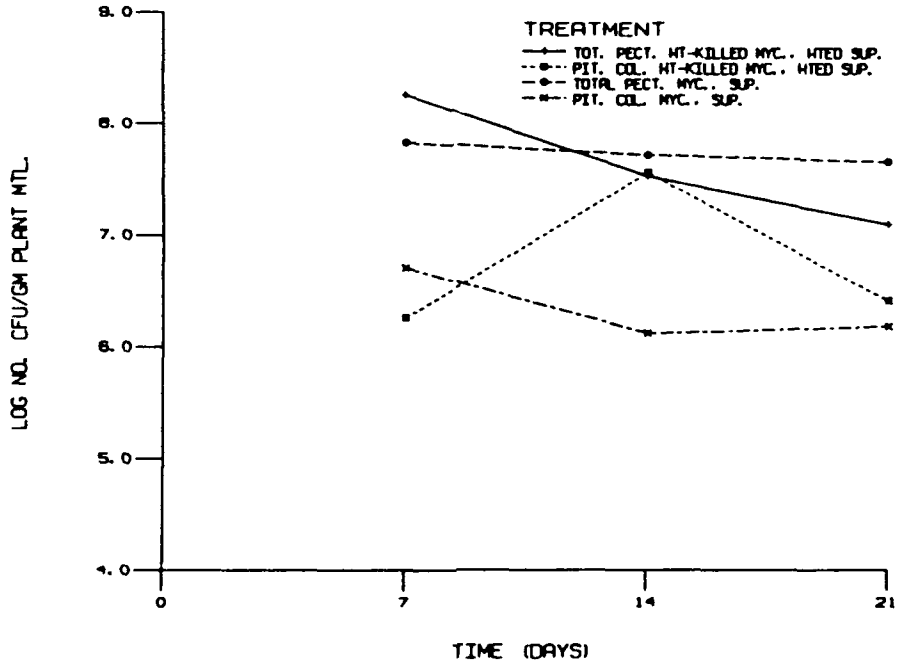

Figure 33. Total and strongly pectinolytic bacterial populations recovered on PA medium from plant tissues variously treated: HT, HTED. = heat, heat-treated; MYC. = *M. t.* mycelia; SUP. = supernatant; PIT. COL. = pitting colonies

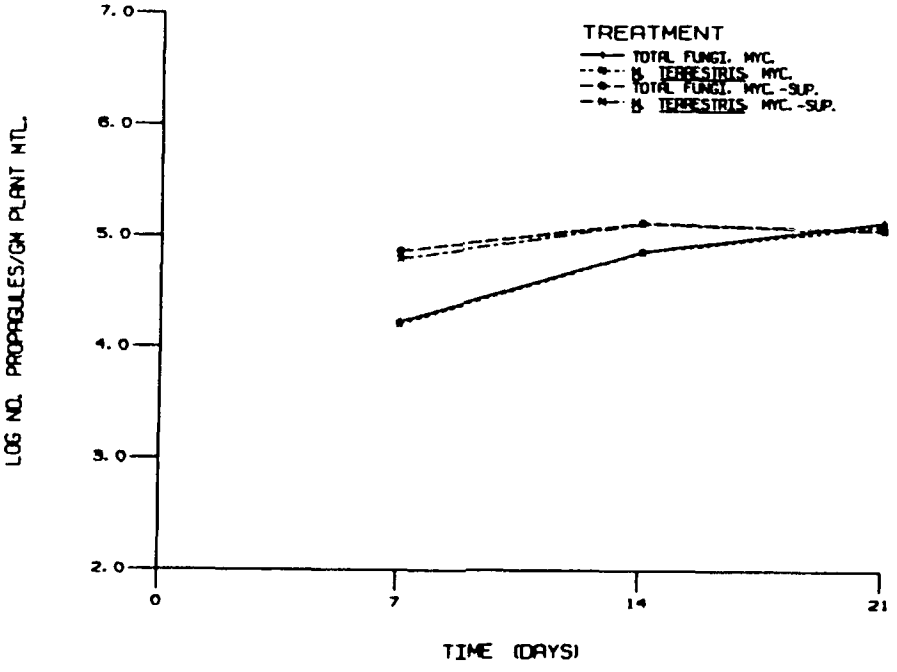

Figure 34. Total fungus and *M. t.* populations recovered on MA medium from plant tissues treated with *M. t.* mycelia alone and *M. t.* mycelia in supernatant

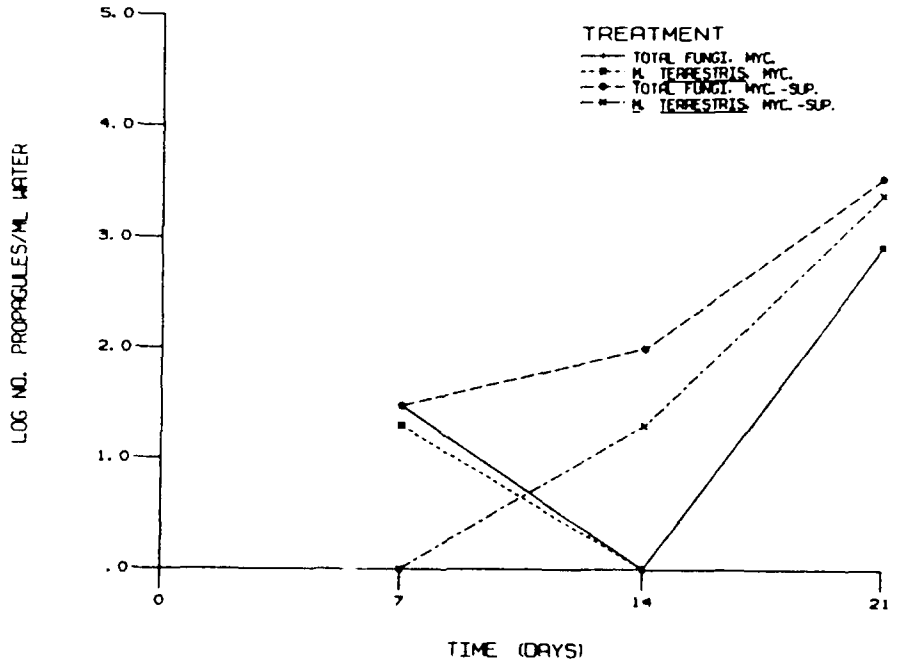

Figure 35. Total fungus and *M. t.* populations recovered on MA medium from water profiles treated with *M. t.* mycelia alone and *M. t.* mycelia in supernatant

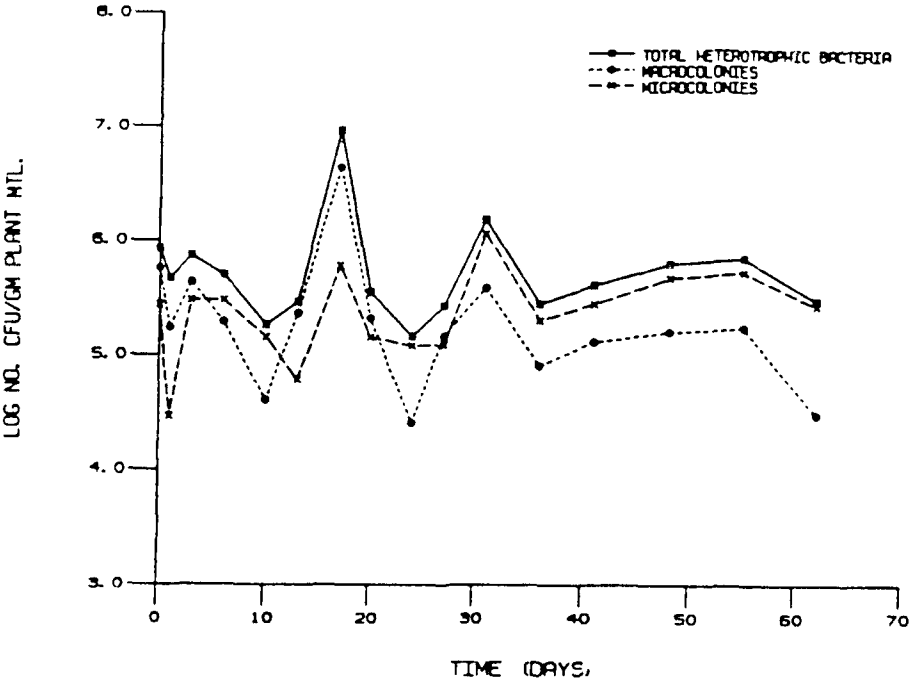

Figure 36. Bacterial populations recovered on TSA medium from untreated plant tissues

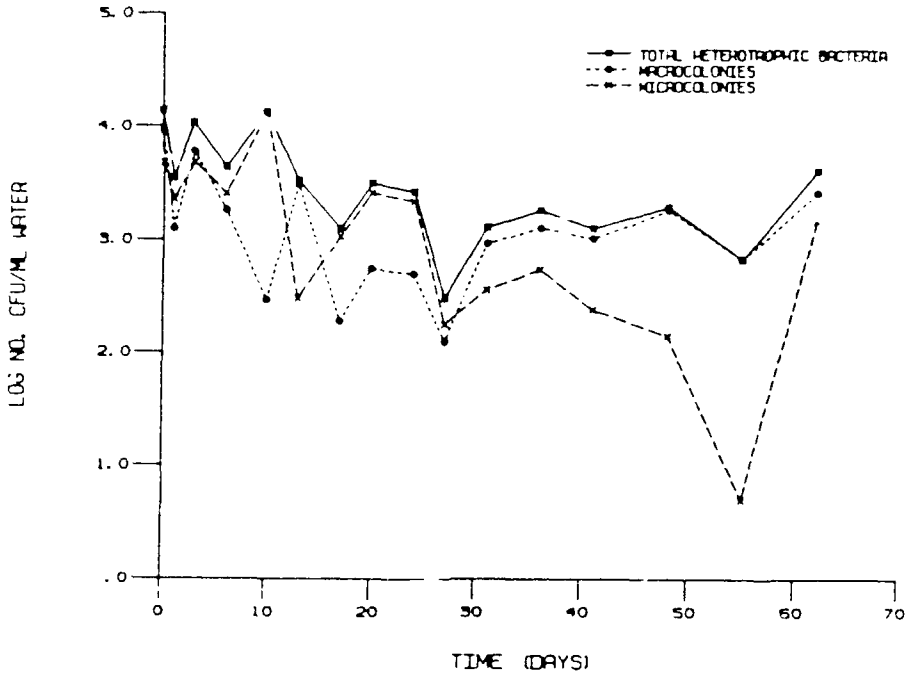

Figure 37. Bacterial populations recovered on TSA medium from untreated water profiles

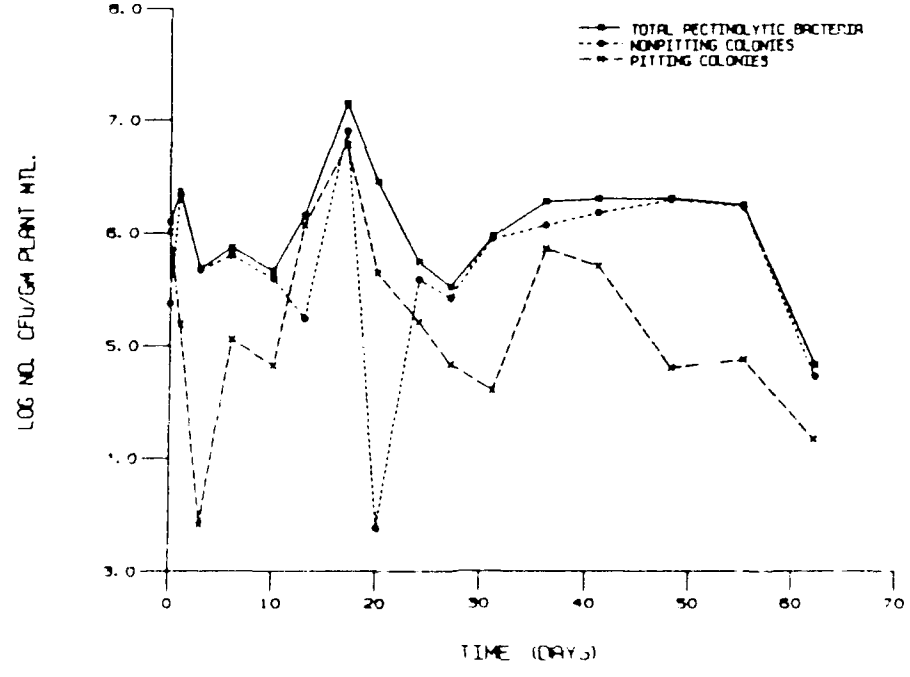

Figure 38. Pectinolytic bacterial populations recovered on PA medium from untreated plant tissues

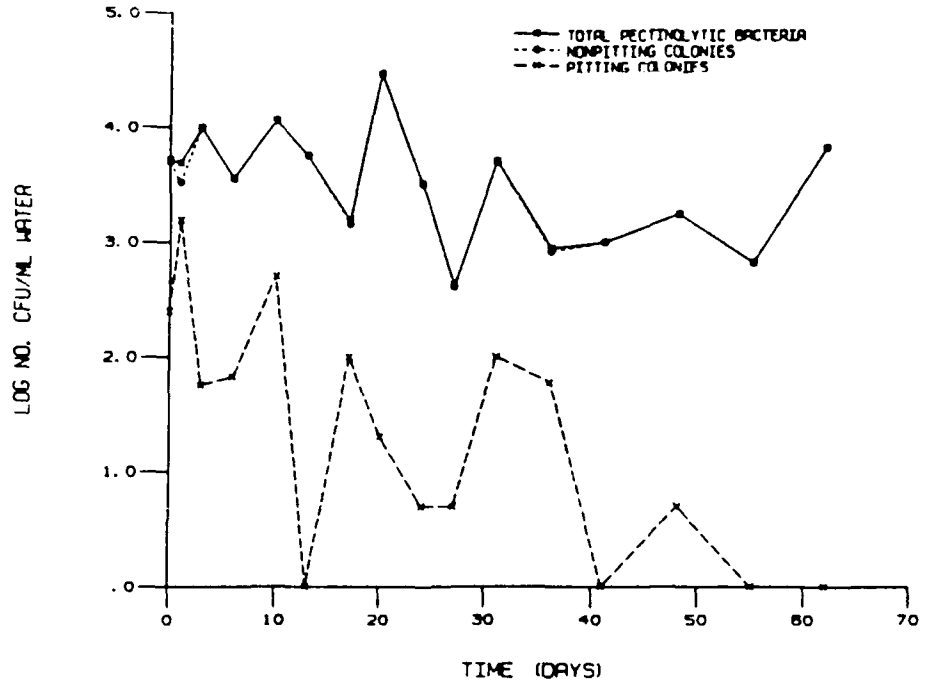

Figure 39. Pectinolytic bacterial populations recovered on PA medium from untreated water profiles

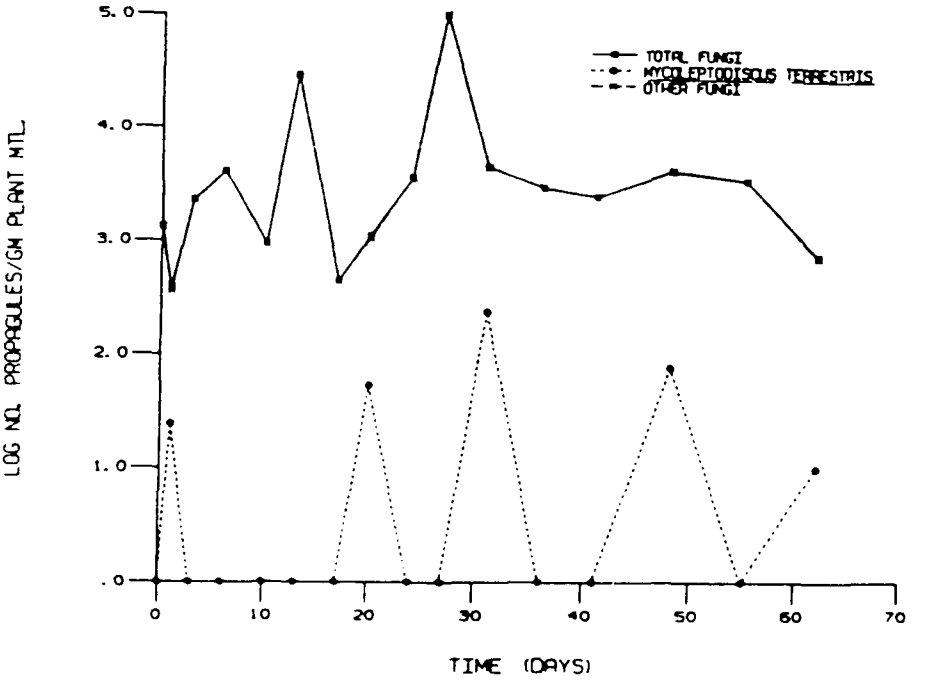

Figure 40. Fungal populations recovered on MA medium from untreated plant tissues

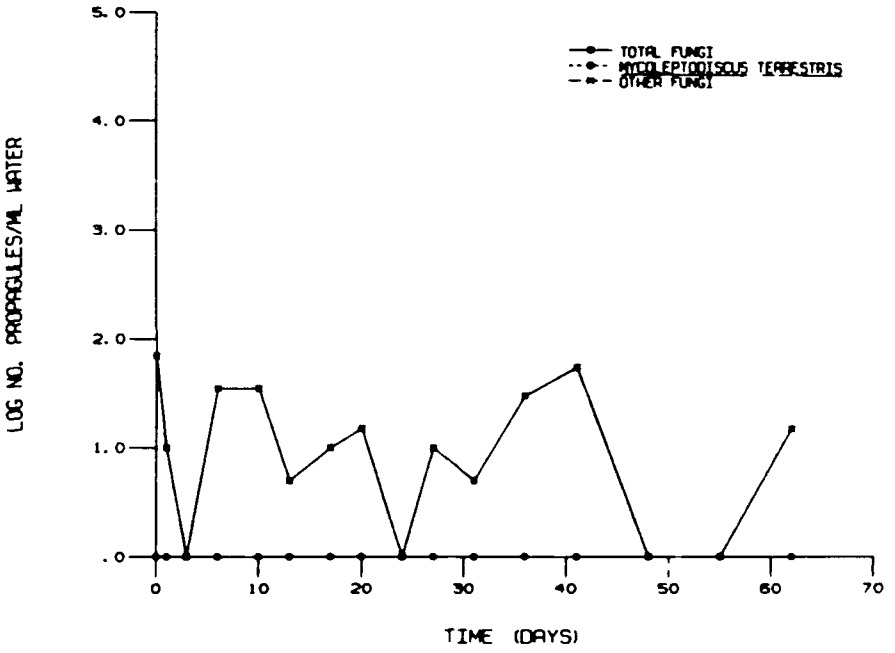

Figure 41. Fungal populations recovered on MA medium from untreated water profiles

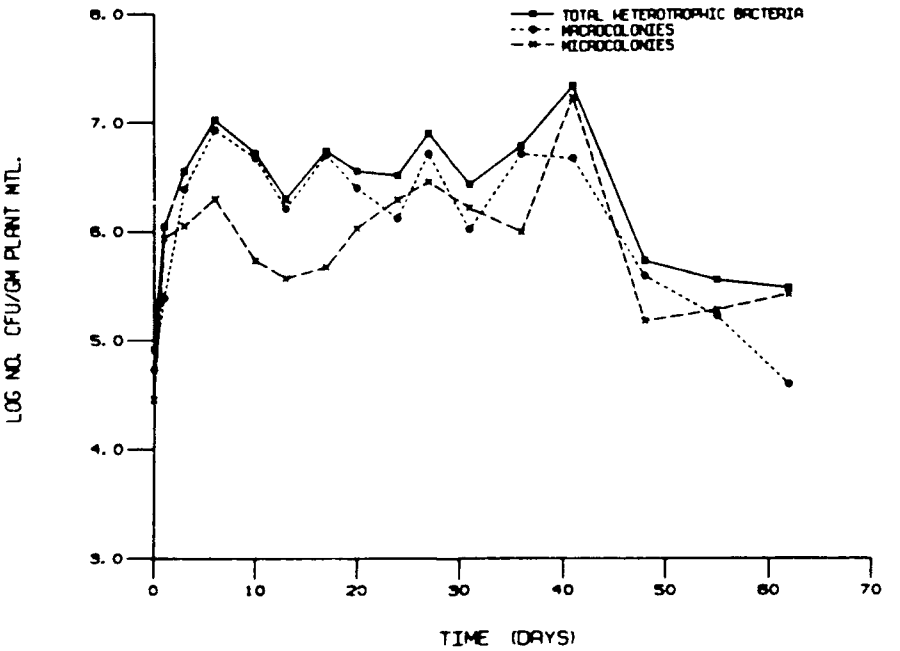

Figure 42. Bacterial populations recovered on TSA medium from *M. t.*-treated plant tissues

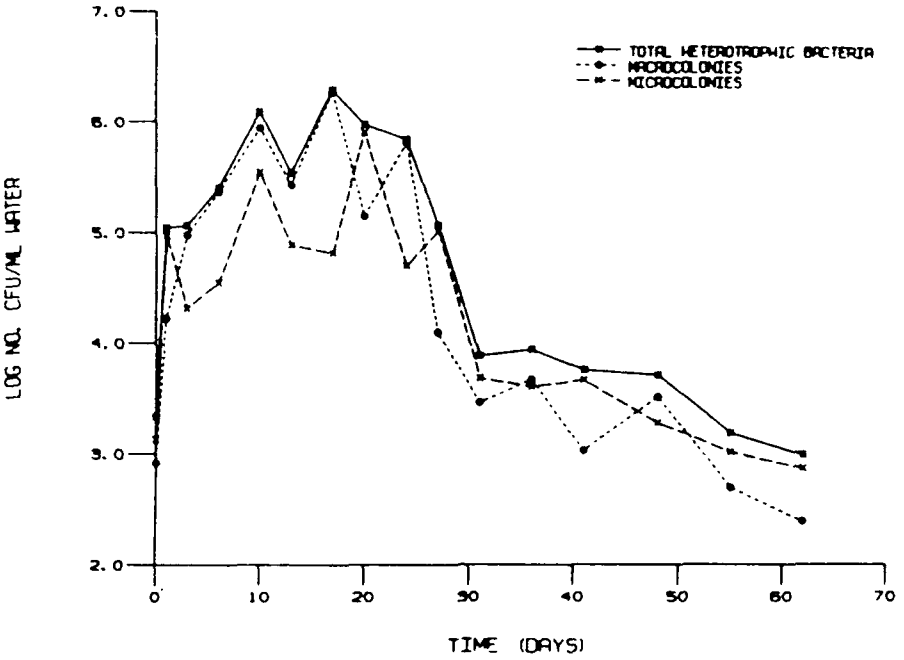

Figure 43. Bacterial populations recovered on TSA medium from *M. t.*-treated water profiles

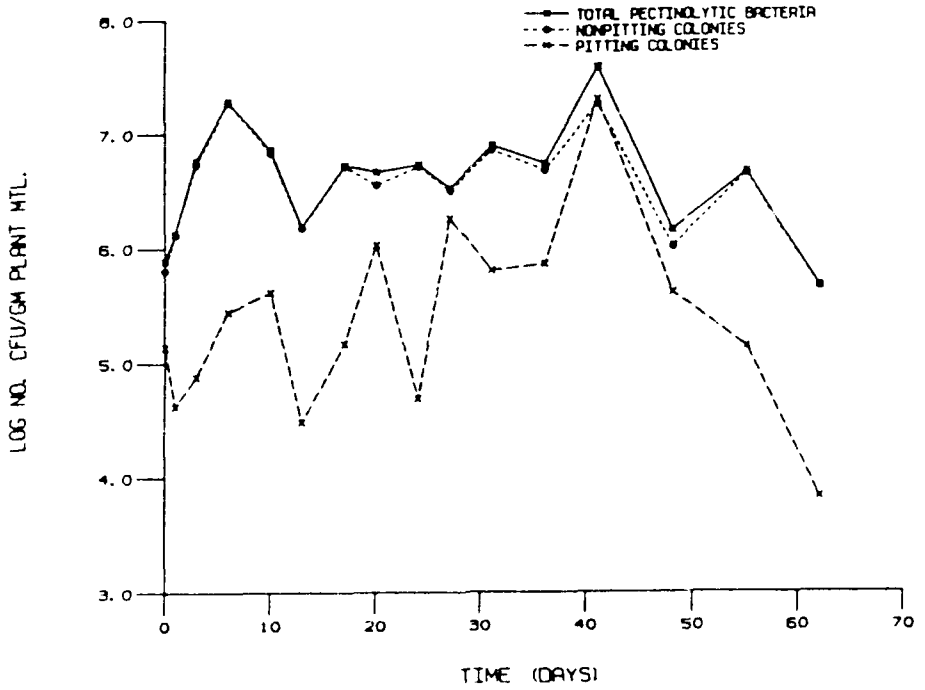

Figure 44. Pectinolytic bacterial populations recovered on PA medium from *M. t.*-treated plant tissues

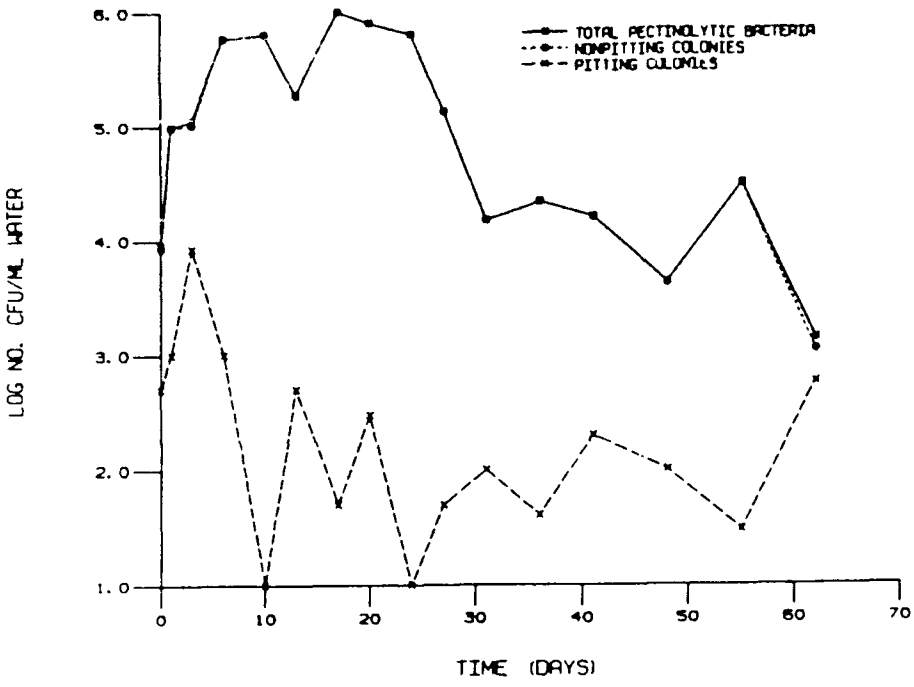

Figure 45. Pectinolytic bacterial populations recovered on PA medium from *M. t.*-treated water profiles

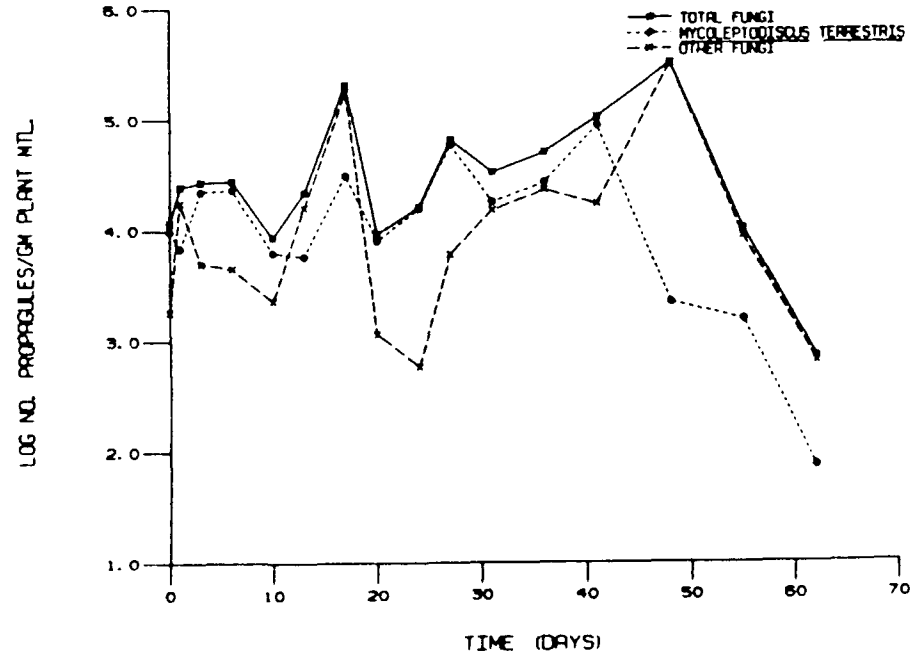

Figure 46. Fungal populations recovered on MA medium from *M. t.*-treated plant tissues

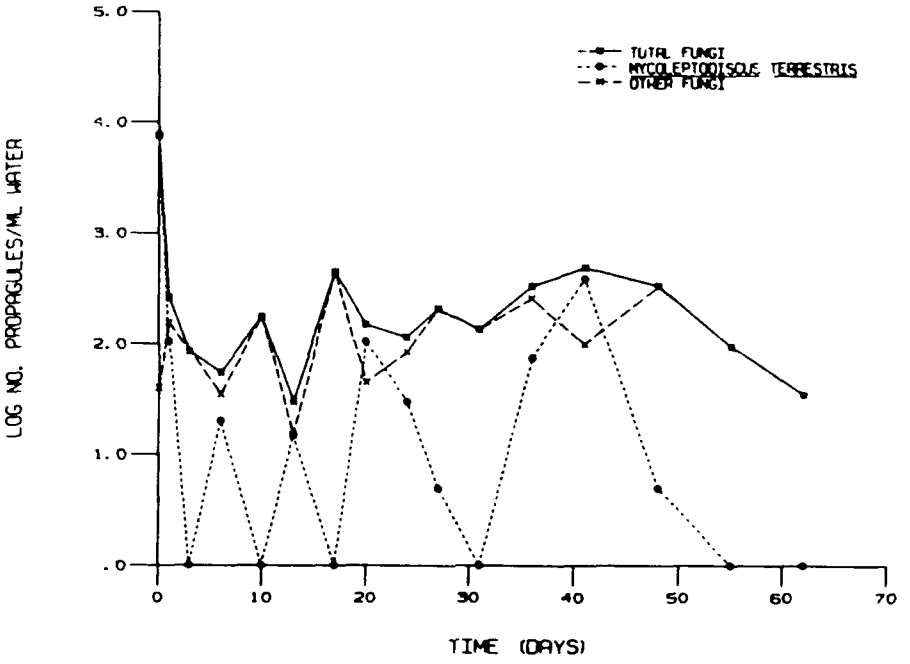

Figure 47. Fungal populations recovered on MA medium from *M. t.*-treated water profiles

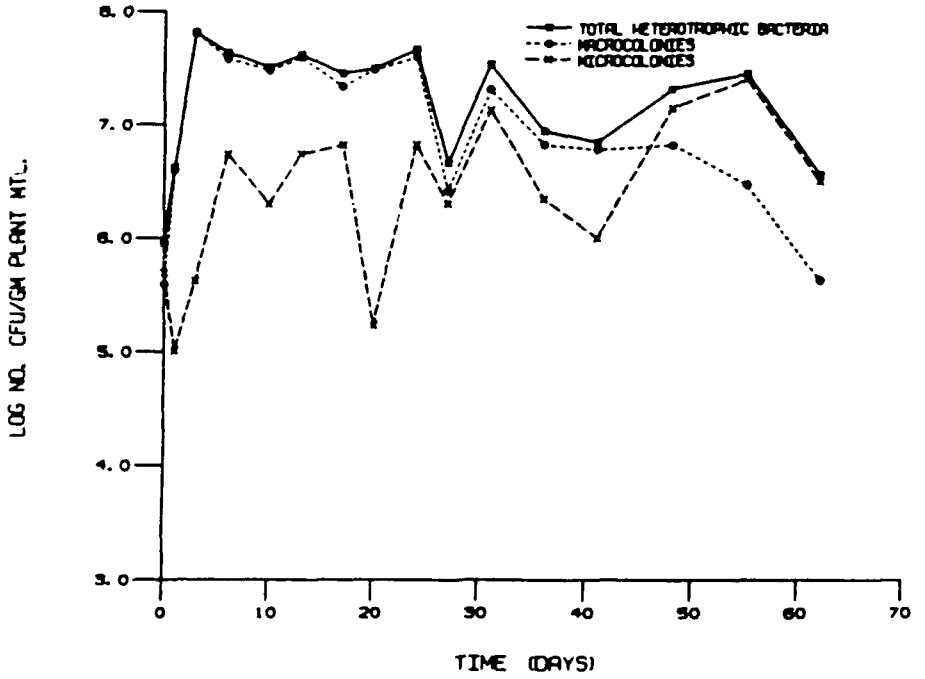

Figure 48. Bacterial populations recovered on TSA medium from *M. t./Bacillus* sp. isolate P8-treated plant tissues

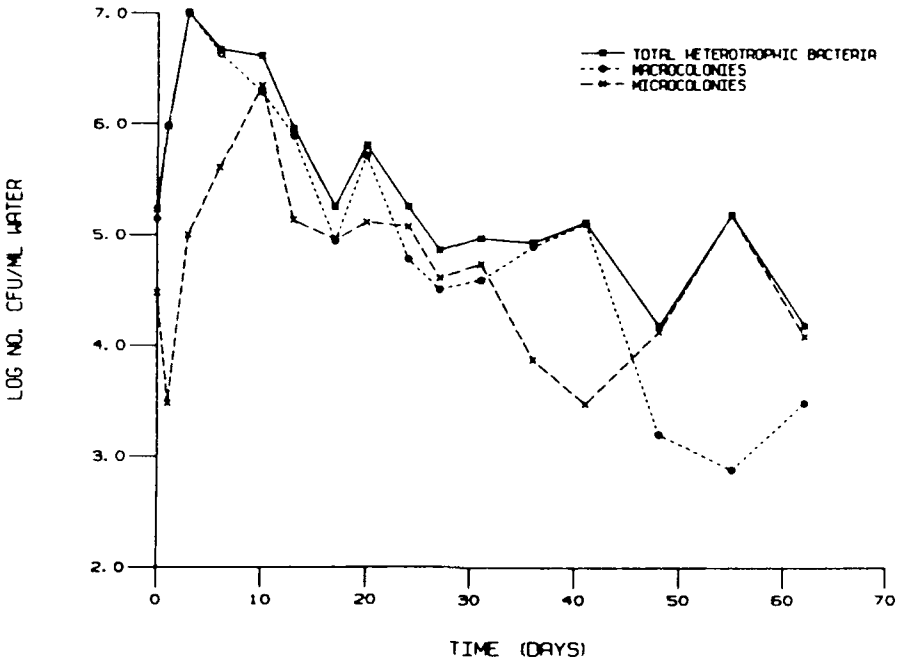

Figure 49. Bacterial populations recovered on TSA medium from *M. t./Bacillus* sp. isolate P8-treated water profiles

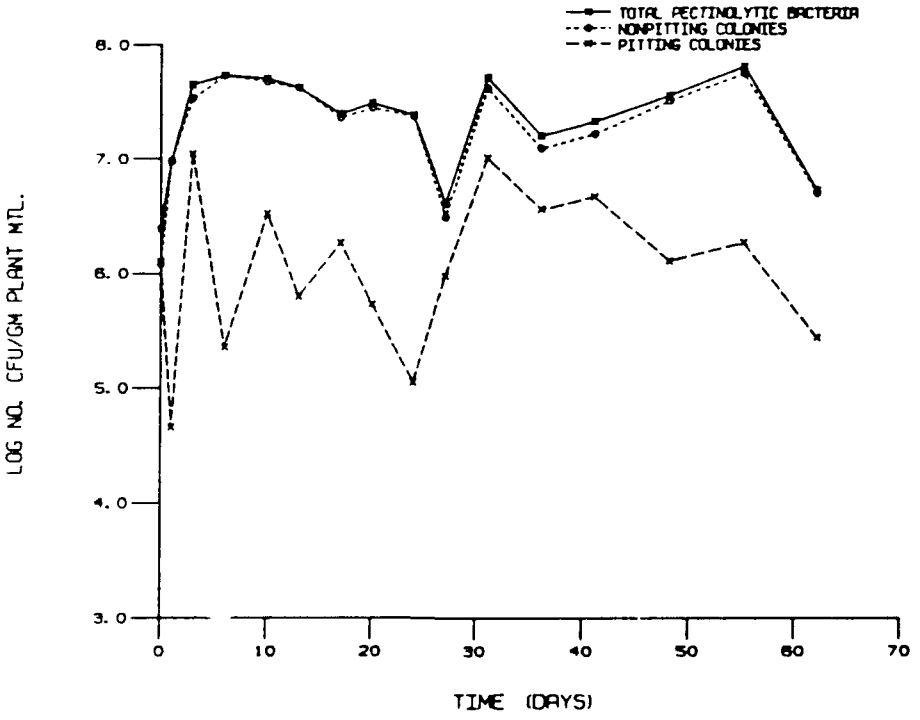

Figure 50. Pectinolytic bacterial populations recovered on PA medium from *M. t./Bacillus* sp. isolate P8-treated plant tissues

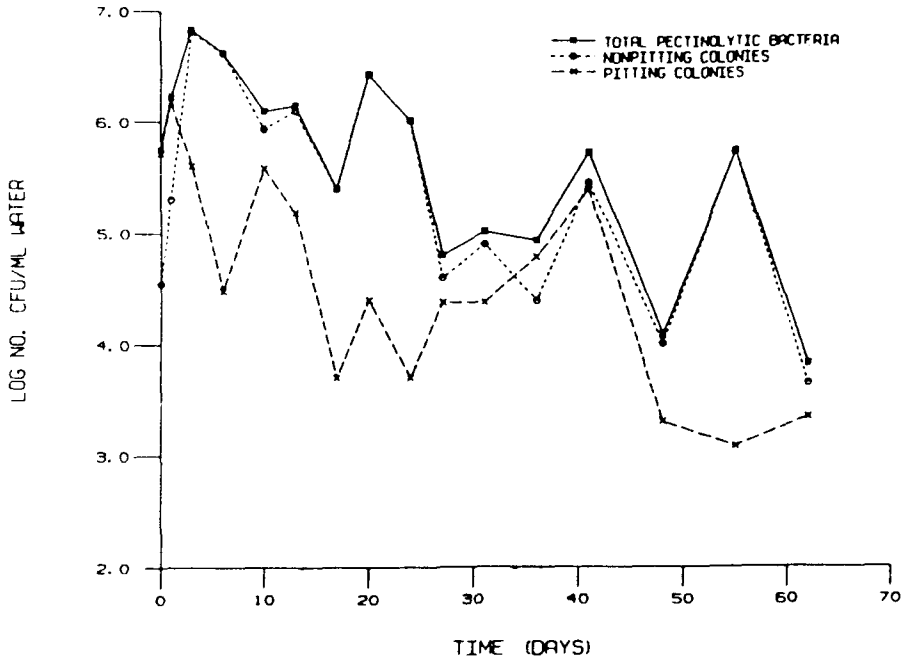

Figure 51. Pectinolytic bacterial populations recovered on PA medium from *M. t./Bacillus* sp. isolate P8-treated water profiles

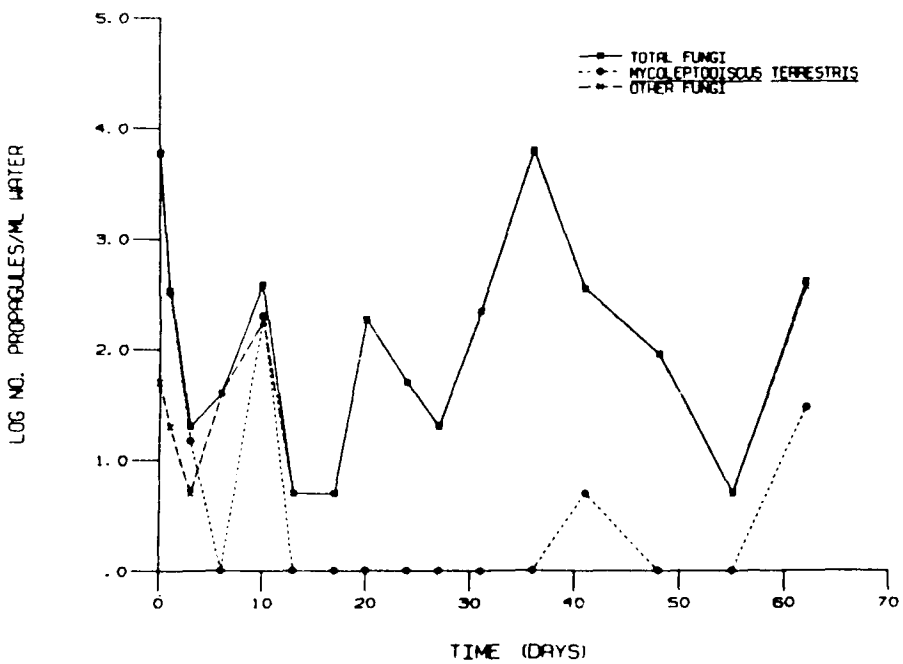

Figure 52. Fungal populations recovered on MA medium from *M. t./Bacillus* sp. isolate P8-treated plant tissues

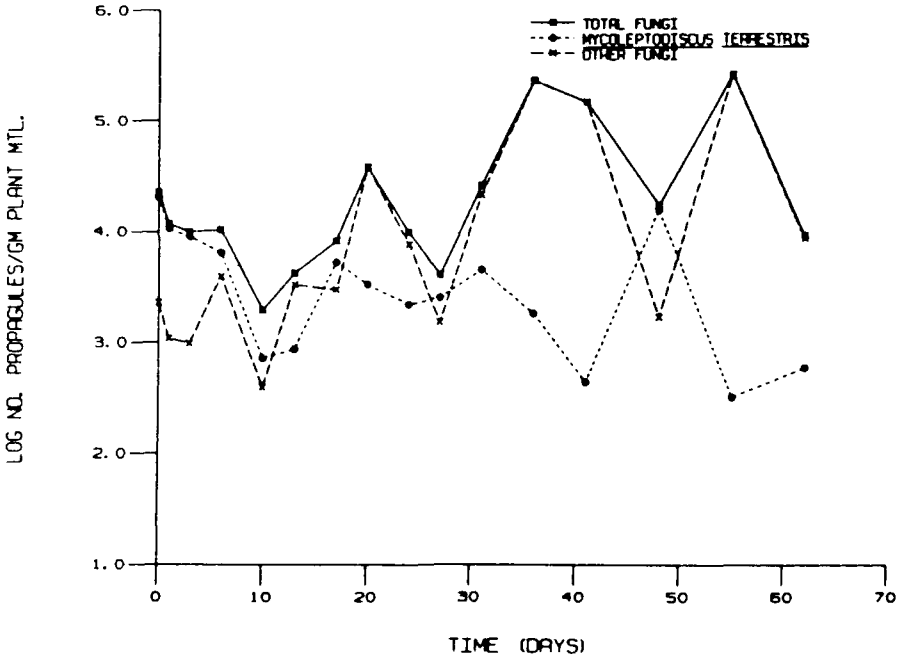

Figure 53. Fungal populations recovered on MA medium from *M. t./Bacillus* sp. isolate P8-treated water profiles

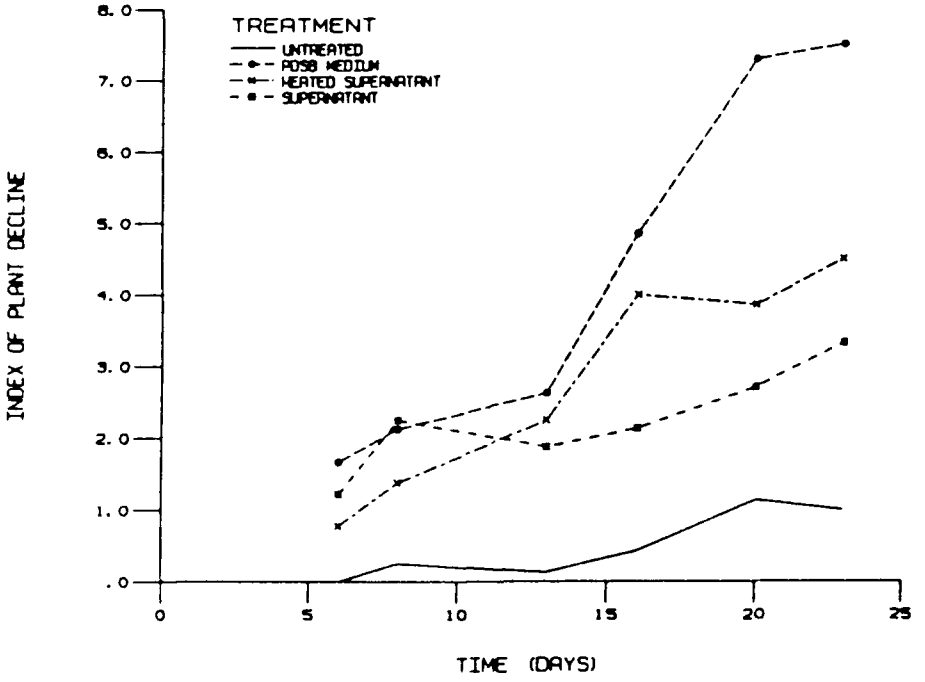

Figure 54. Visual evaluation of *M. spicatum* in untreated jars and jars treated with PDSB medium, heated and unheated supernatant

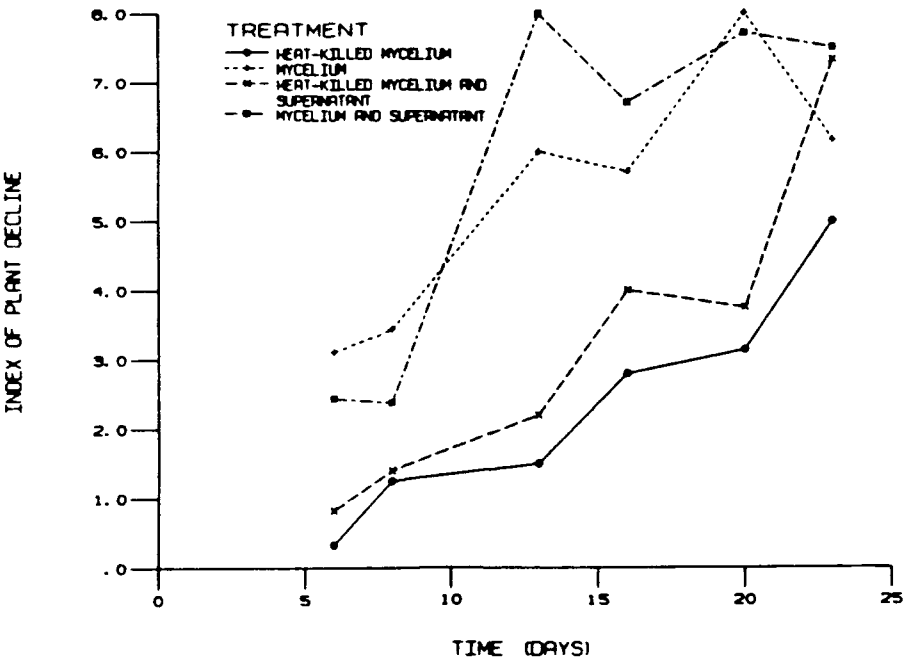

Figure 55. Visual evaluation of *M. spicatum* in jars treated with heat-killed *M. t.* mycelia, heat-killed *M. t.* mycelia in heat-treated supernatant, live *M. t.* mycelia, and live *M. t.* mycelia in untreated supernatant

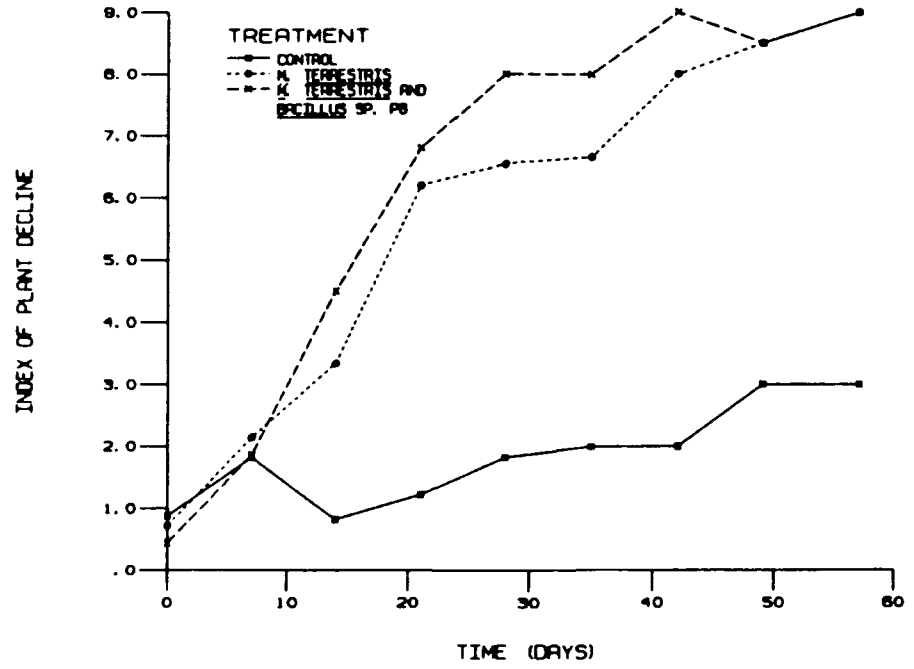

Figure 56. Visual evaluation of *M. spicatum* in control (untreated) jars and jars treated with *M. t.* and with *M. t.* and *Bacillus* sp. isolate P8

a. Uninoculated control

b. Inoculated with 1 ml *M. t.* culture

c. Inoculated with 3 ml *M. t.* culture

d. Inoculated with 5 ml *M. t.* culture

Figure 57. Representative samples of *M. spicatum* from dose response test in jars 16 days after inoculation

Figure 58. *Myriophyllum spicatum* samples from dose response jar experiment removed from cups to expose roots

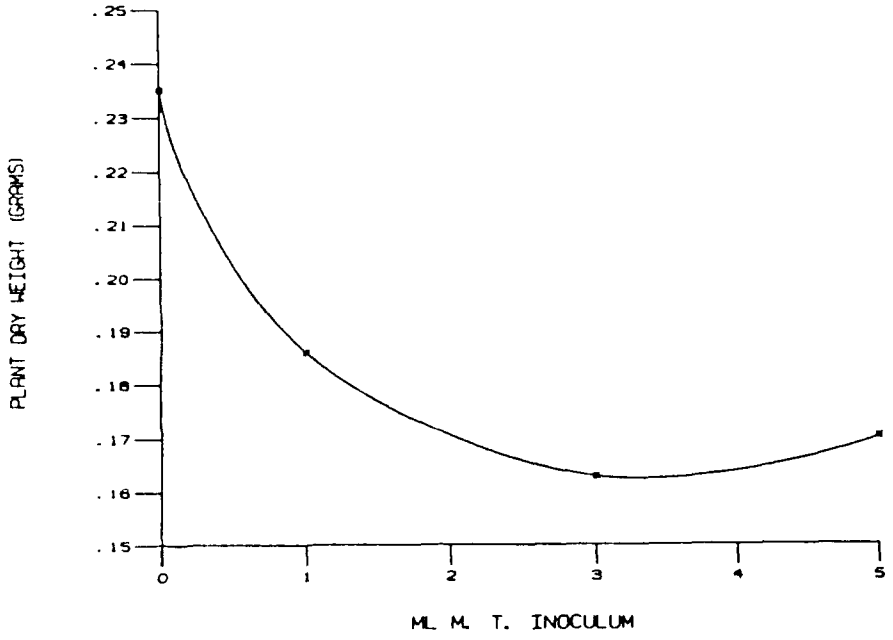

Figure 59. Biomass of *M. spicatum* from dose response jar experiment after 16 days

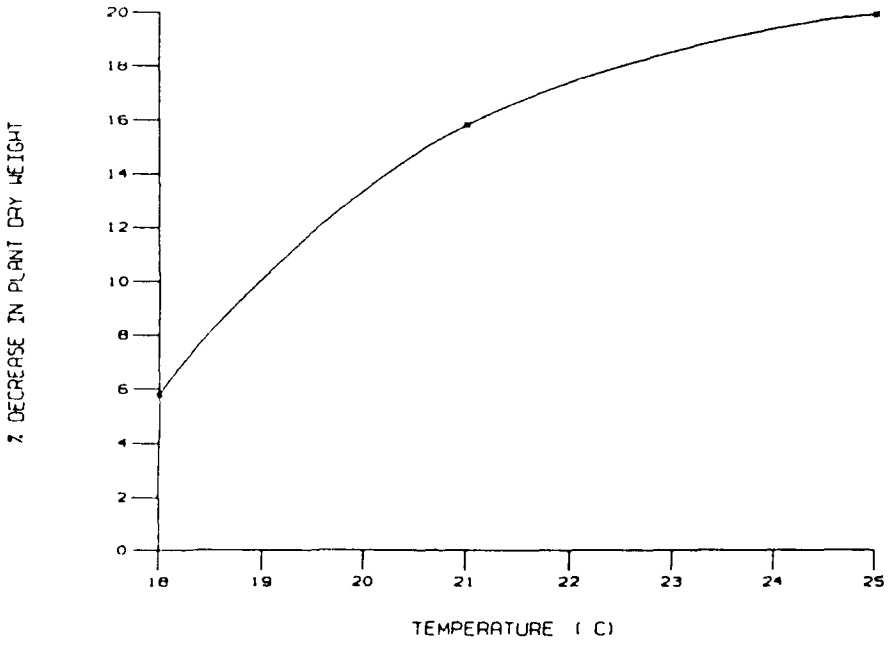

Figure 60. Biomass results expressed as percent decrease in dry weight of treated versus control of *M. spicatum* incubated at various temperatures 16 days after inoculation

Figure 61. Section of young leaf of uninoculated surface of *M. spicatum* (1,800×). Note relative paucity of microbial populati ns

Figure 62. Section of young stem of uninoculated surface of *M. spicatum* (1,800×). Note relative paucity of microbial populations

Figure 63. Section of old leaf of uninoculated surface of *M. spicatum* (1,800×). Note relatively little increase in resident microflora with age

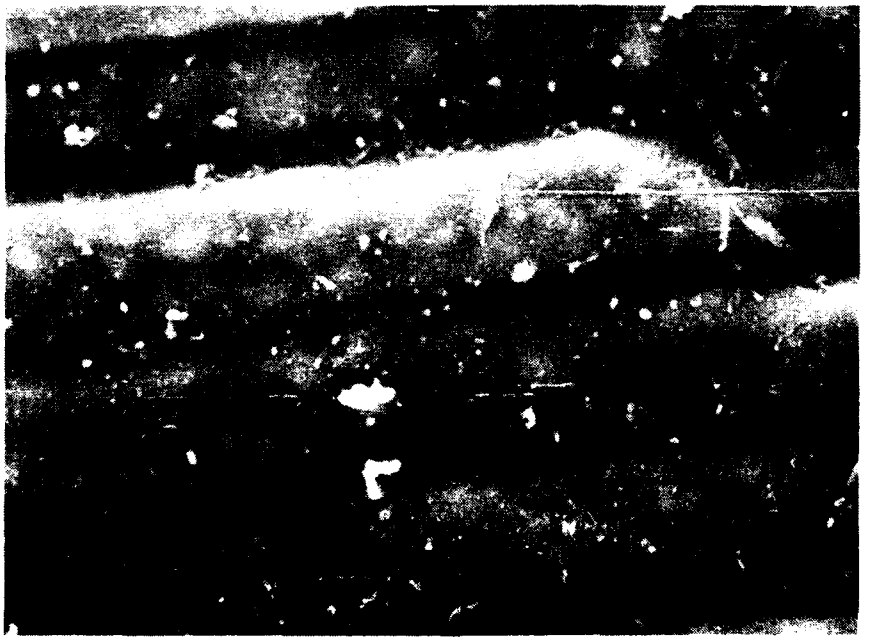

Figure 64. Section of old stem of uninoculated surface of *M. spicatum* (1,800×). Note relatively little increase in resident microflora with age

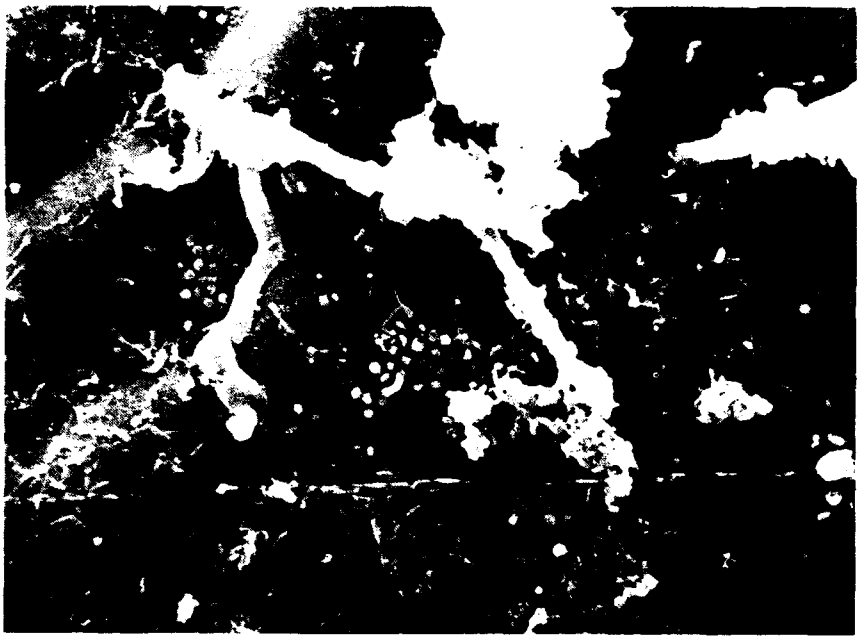

Figure 65. Section of young leaf of *M. t.*-treated *M. spicatum* (1,800×). Note presence of fungal mycelium

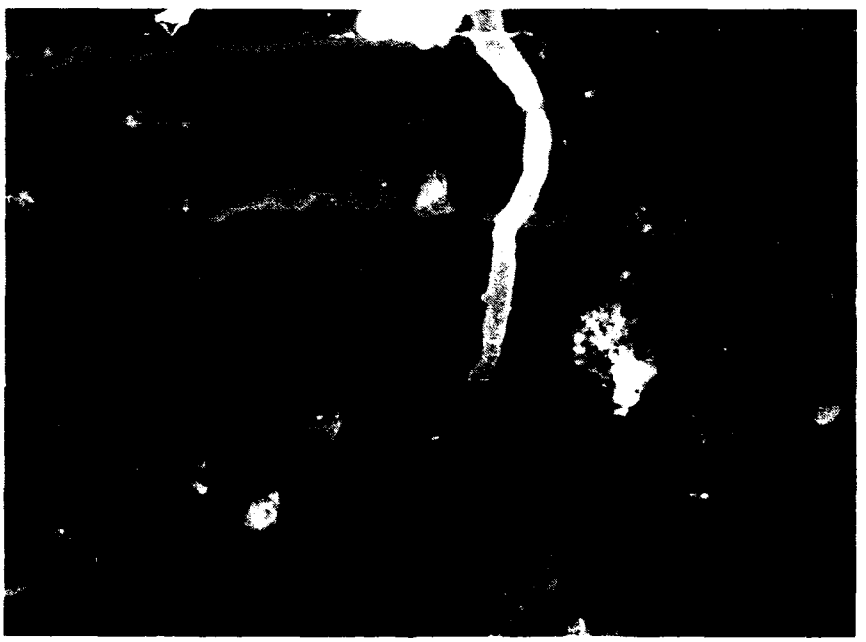

Figure 66. Section of young stem of *M. t.*-treated *M. spicatum* (1,800×). Note presence of fungal mycelium

Figure 67. Section of old leaf of *M. t.*-treated *M. spicatum* (1,800×). Note increase of resident bacterial populations on older leaf tissues

Figure 68. Section of old stem of *M. t.*-treated *M. spicatum* (1,800×). Note increase of resident bacterial populations on older leaf tissues

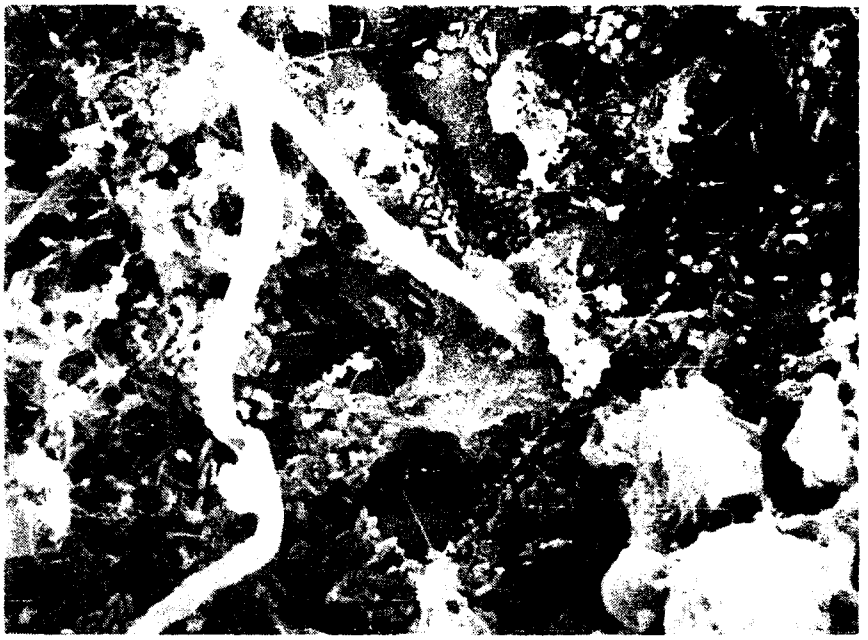

Figure 69. Section of young leaf of *Bacillus* sp. isolate P8/*M. t.*-treated *M. spicatum* (1,800×). Note heavy infestation of bacterial populations embedded in xanthan carrier and presence of fungal mycelium

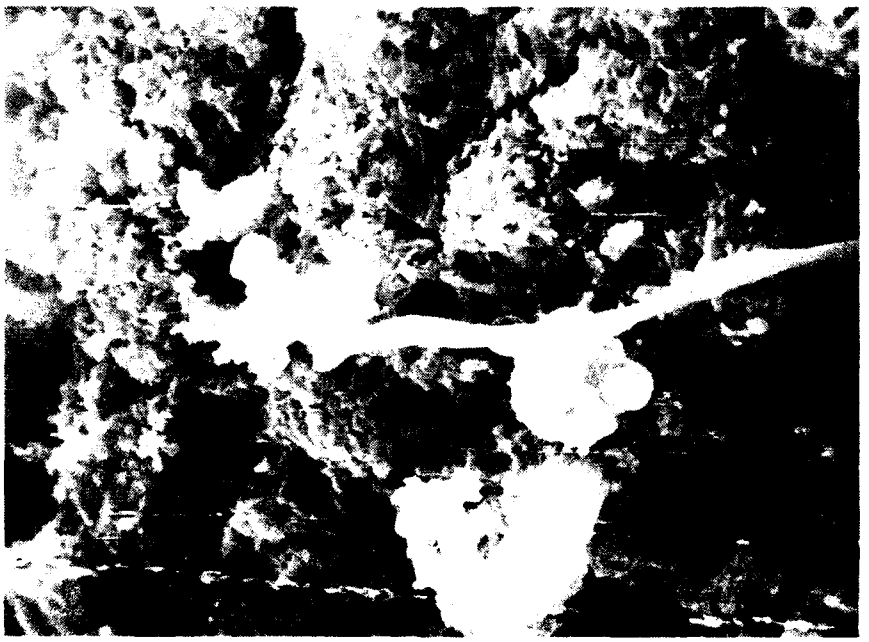

Figure 70. Section of young stem of *Bacillus* sp. isolate P8/*M. t.*-treated *M. spicatum* (1,800×). Note heavy infestation of bacterial populations embedded in xanthan carrier and presence of fungal mycelium

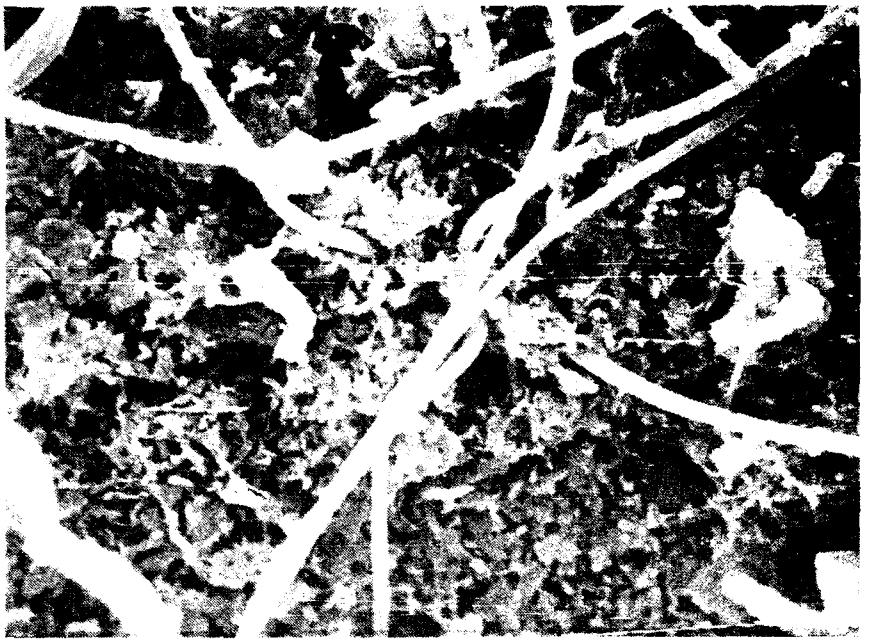

Figure 71. Section of old leaf of *Bacillus* sp. isolate P8/*M. t.*-treated *M. spicatum* (1,800×). Note heavy infestation of bacterial populations embedded in xanthan carrier and presence of fungal mycelium

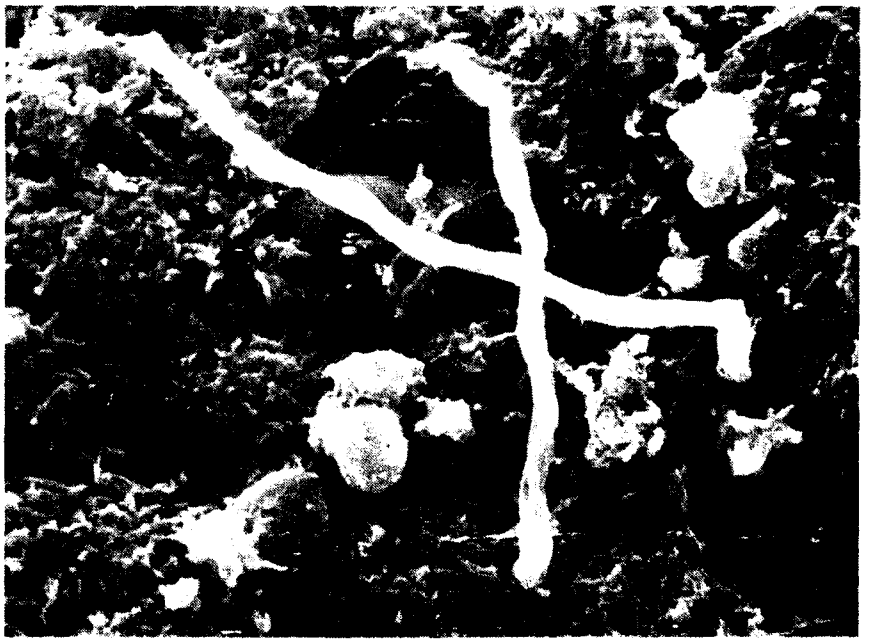

Figure 72. Section of old stem of *Bacillus* sp. isolate P8/*M. t.*-treated *M. spicatum* (1,800×). Note heavy infestation of bacterial populations embedded in xanthan carrier and presence of fungal mycelium

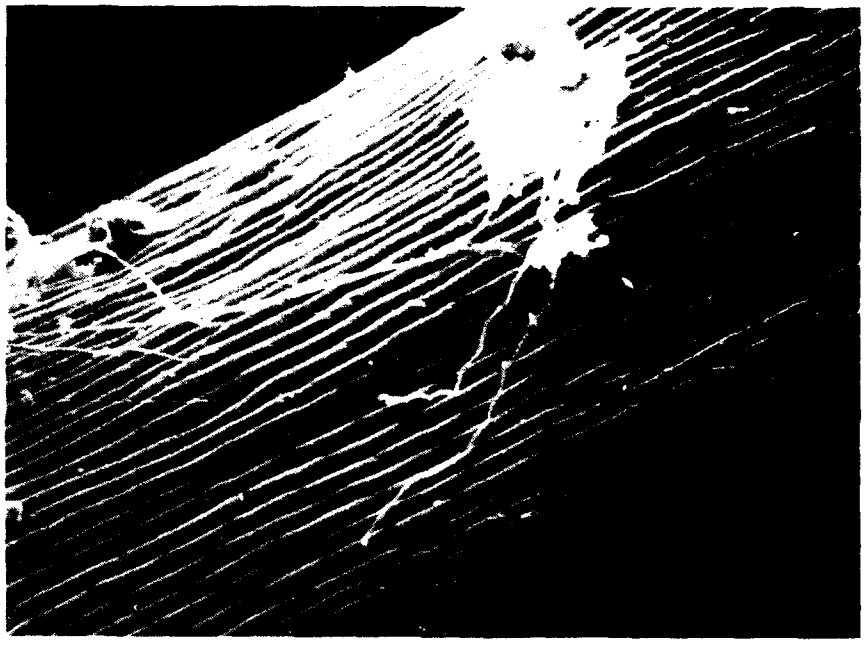

Figure 73. Entry site of *M. t.* into young stem of *M. spicatum* (350×). Note fungal inoculum infection source extending from it

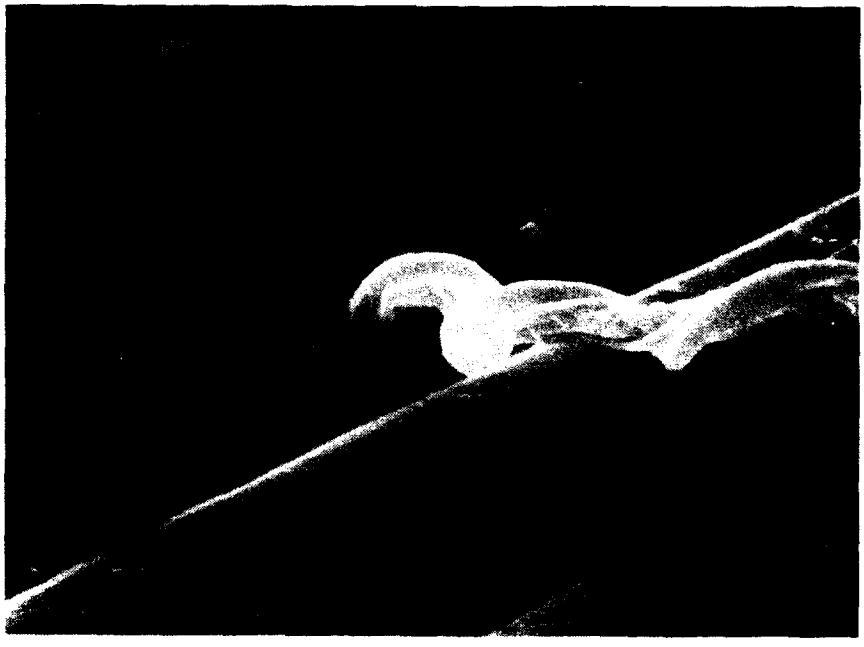

Figure 74. Entry site of *M. t.* into young stem of *M. spicatum* (4,000×). Note penetration of *M. t.* into plant tissue

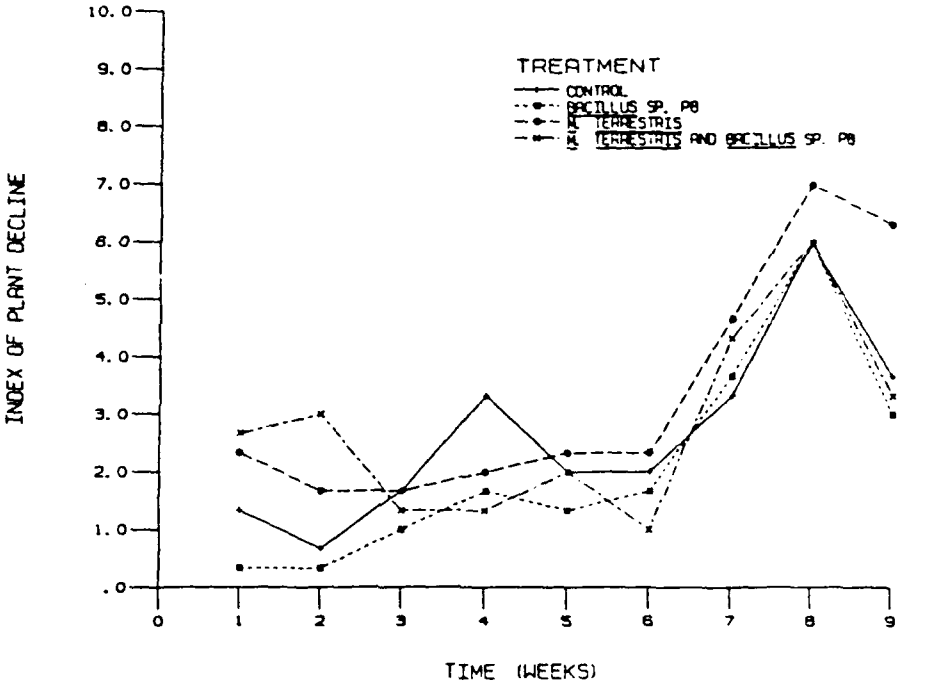

Figure 75. Visual evaluation of condition of *M. spicatum* in pools after microbial treatments. Index represents averages of three replicates

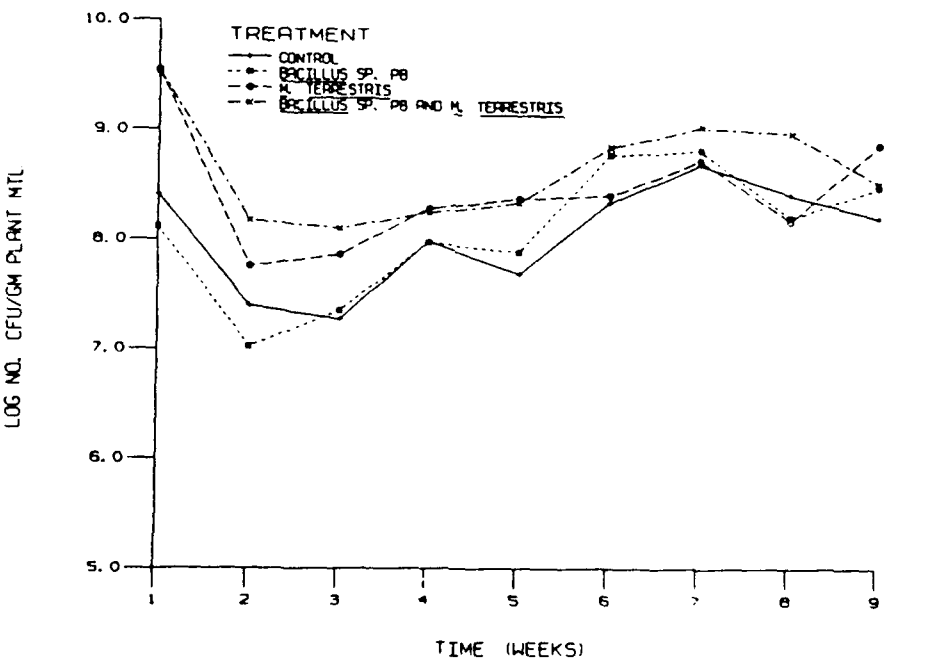

Figure 76. Bacterial populations recovered on TSA medium from control (untreated) and treated *M. spicatum* tissues in pool experiment

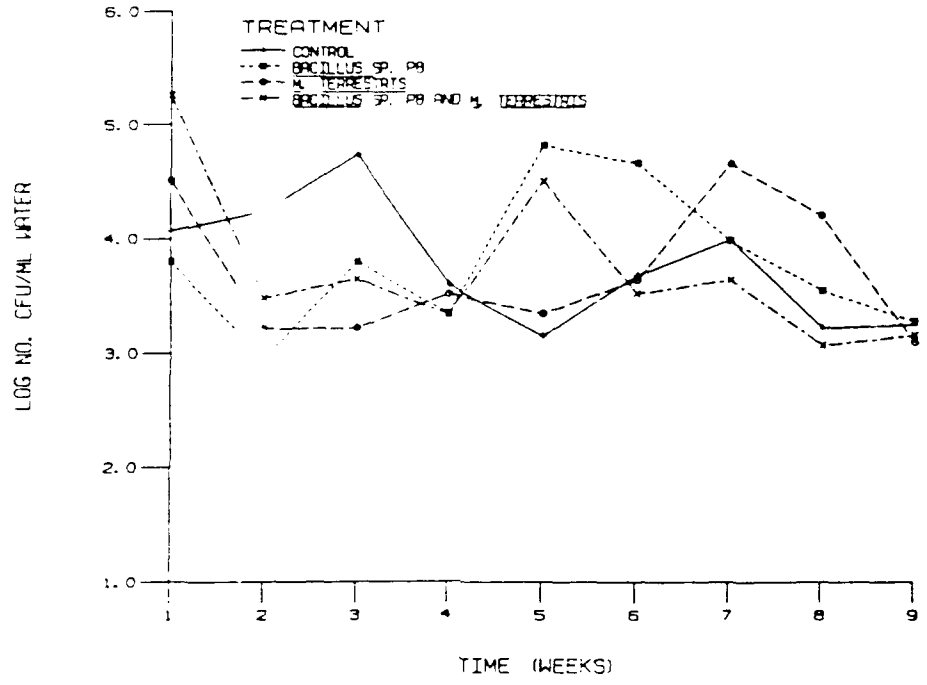

Figure 77. Bacterial populations recovered on TSA medium from control (untreated) and treated water profiles in pool experiment

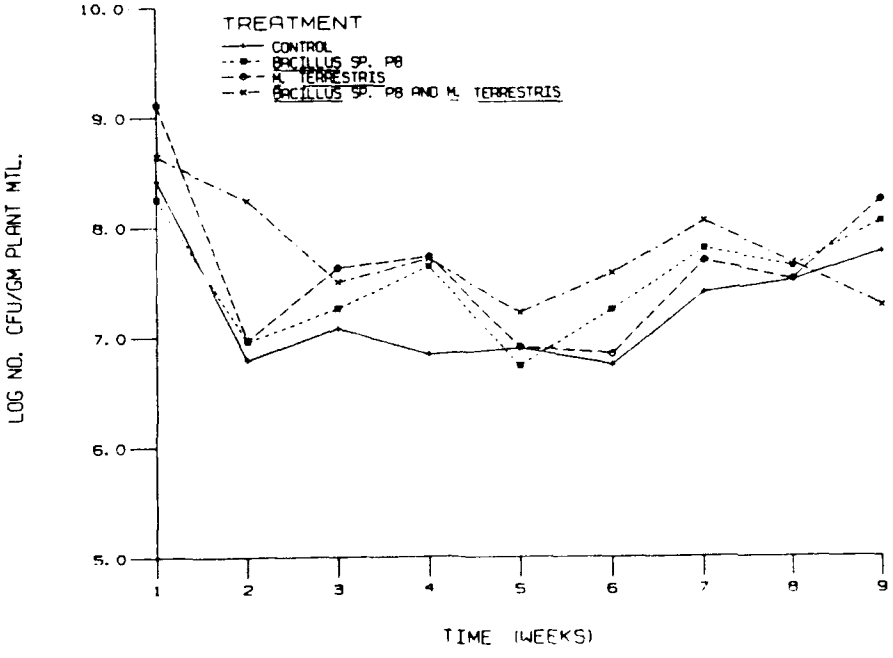

Figure 78. Total and strongly pectinolytic bacterial populations recovered on PA medium from control (untreated) and treated *M. spicatum* tissues in pool experiment

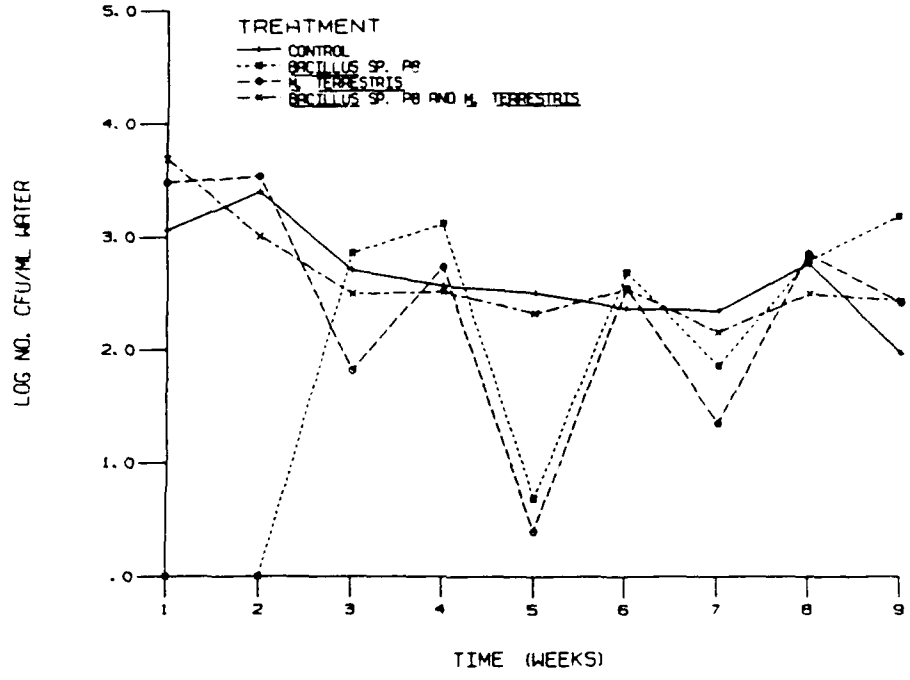

Figure 79. Total and strongly pectinolytic bacterial populations recovered on PA medium from control (untreated) and treated water profiles in pool experiment

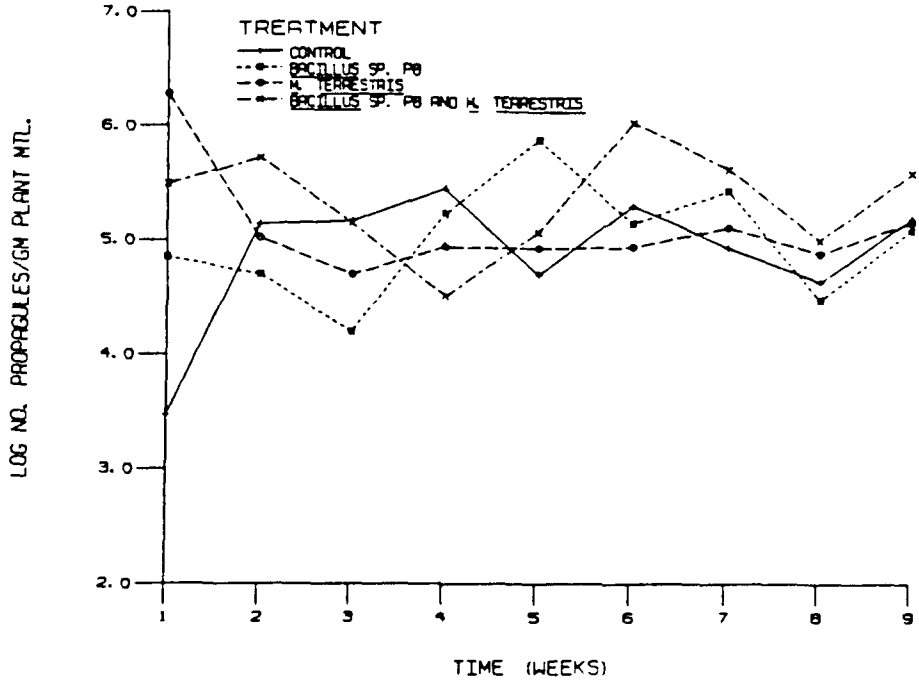

Figure 80. Fungal populations recovered on MA medium from control (untreated) and treated *M. spicatum* tissues in pool experiment

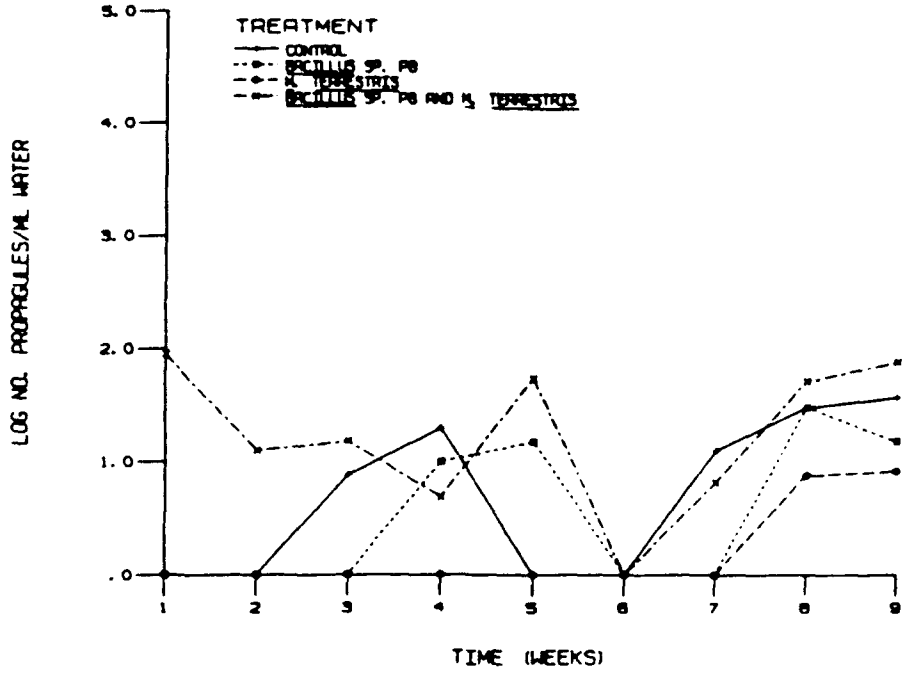

Figure 81. Fungal populations recovered on MA medium from control (untreated) and treated water profiles in pool experiment

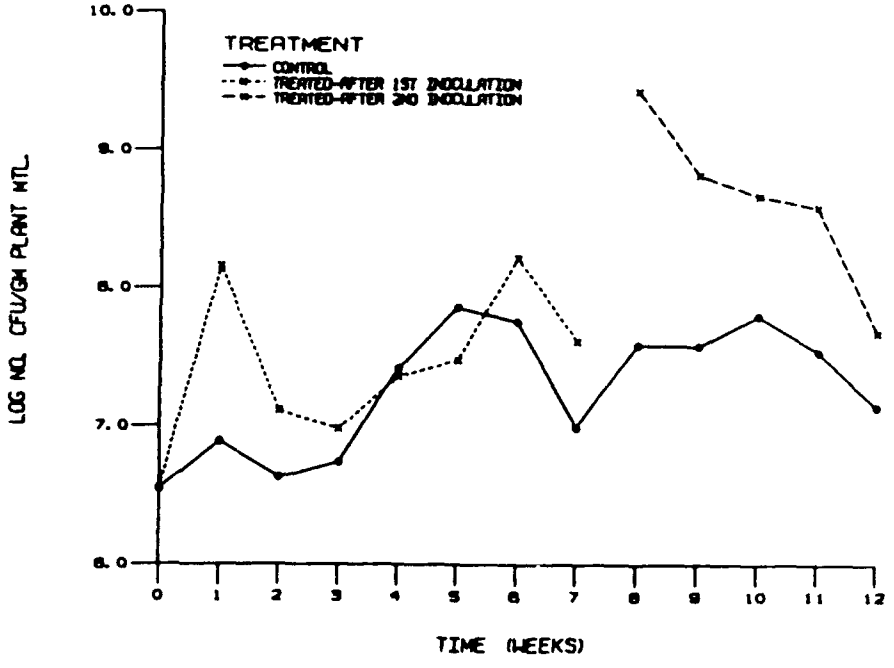

Figure 82. Bacterial populations recovered on TSA medium from control (untreated) and treated *M. spicatum* tissues in 1985 field experiment

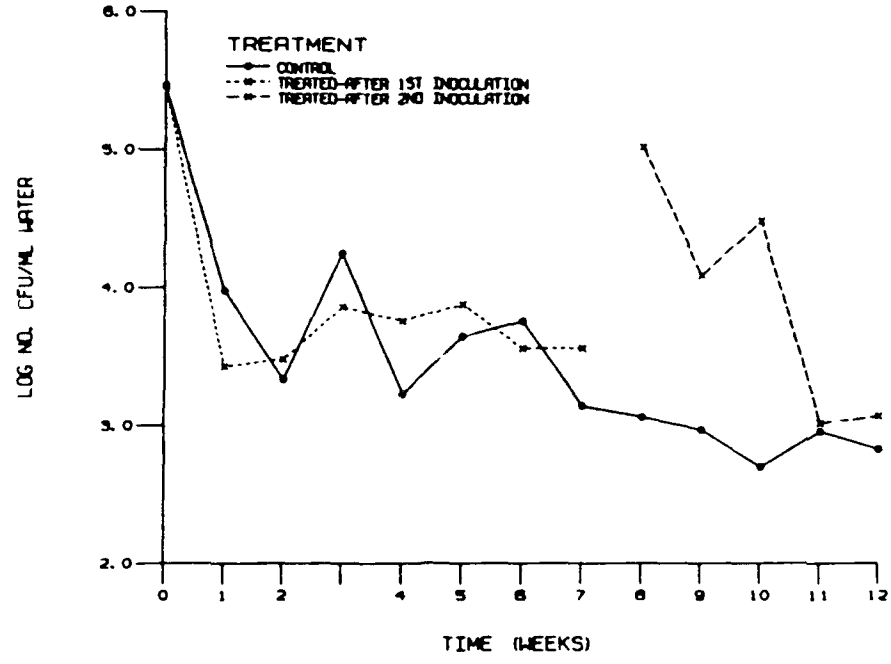

Figure 83. Bacterial populations recovered on TSA medium from water profiles of control (untreated) and treated plots containing *M. spicatum* in 1985 field experiment

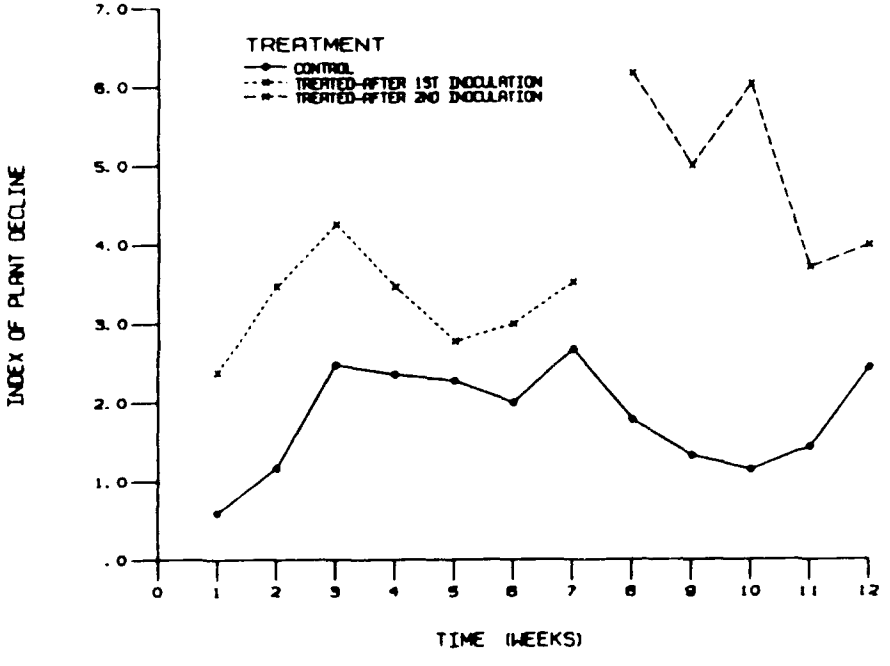

Figure 84. Visual evaluation of condition of *M. spicatum* in 1985 field experiment from control (untreated) and treated (with *M. t.* and *Bacillus* sp. isolate P8) plots. Index represents averages of observations from nine replicates

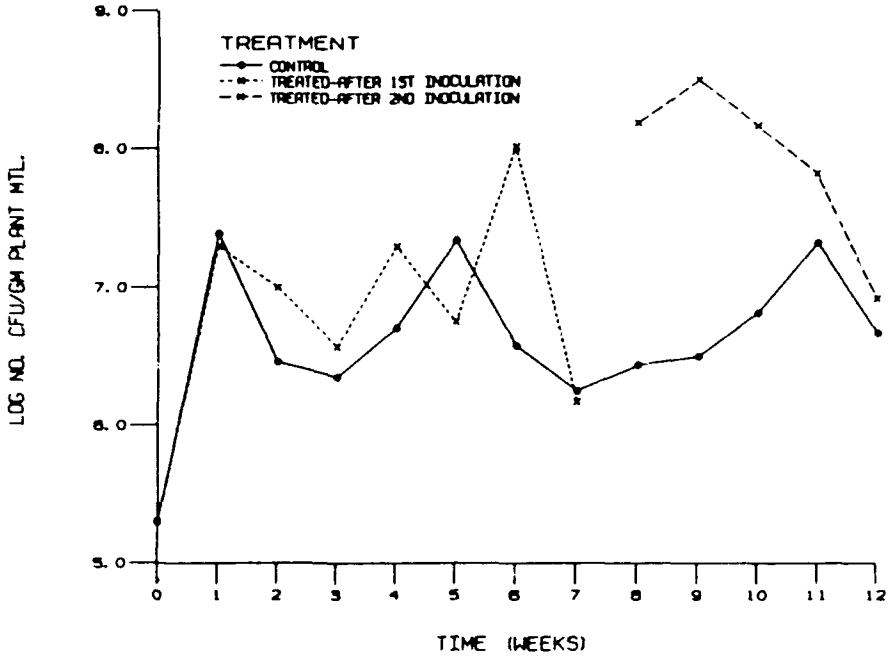

Figure 85. Populations of strongly pectinolytic bacteria recovered on PA medium from control (untreated) and treated *M. spicatum* tissues in 1985 field experiment

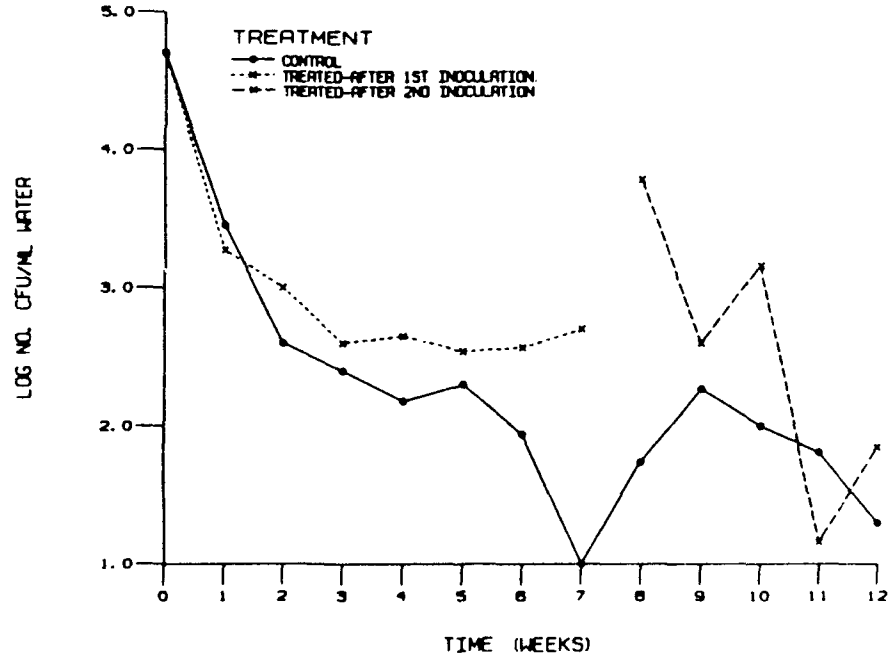

Figure 86. Populations of strongly pectinolytic bacteria recovered on PA medium from water profiles of control (untreated) and treated plots containing *M. spicatum* in 1985 field experiment

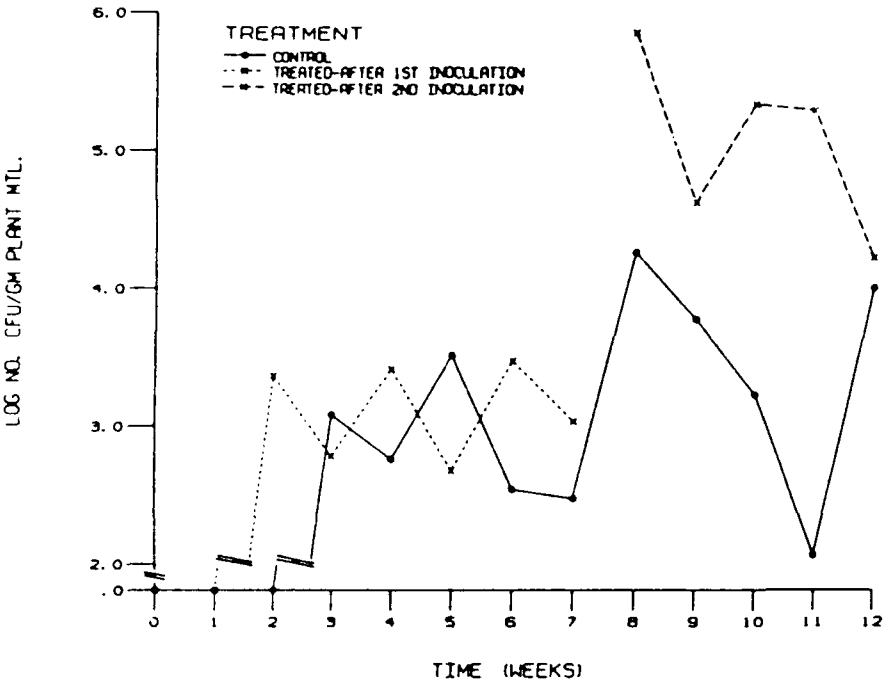

Figure 87. Fungal populations recovered on MA medium from control (untreated) and treated *M. spicatum* tissues in 1985 field experiment

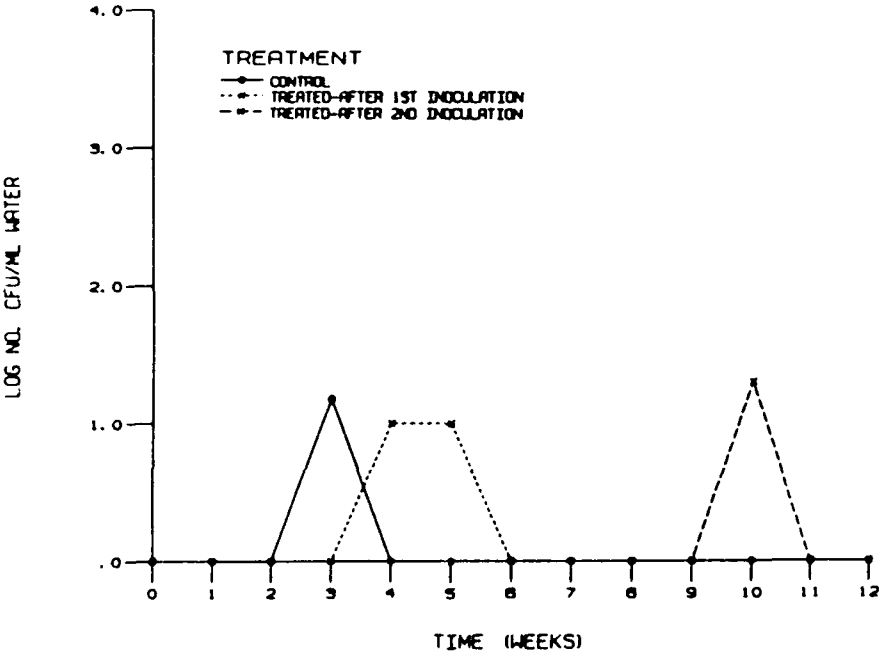

Figure 88. Fungal populations recovered on MA medium from water profiles of control (untreated) and treated plots containing *M. spicatum* in 1985 field experiment

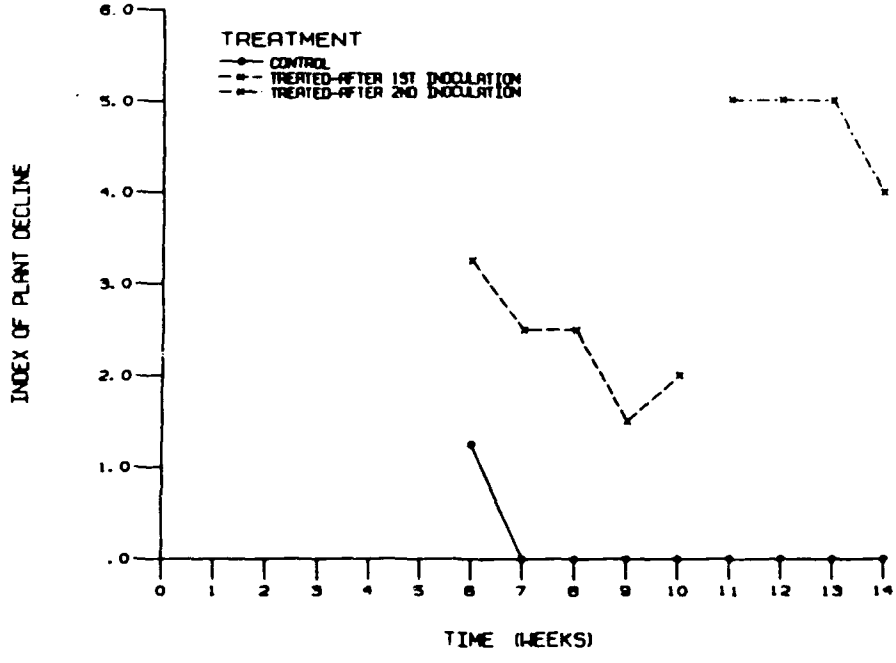

Figure 89. Visual evaluation of condition of *M. spicatum* in 1986 field experiment from control (untreated) plot and plot treated with *M. t.* and *Bacillus* isolate P8

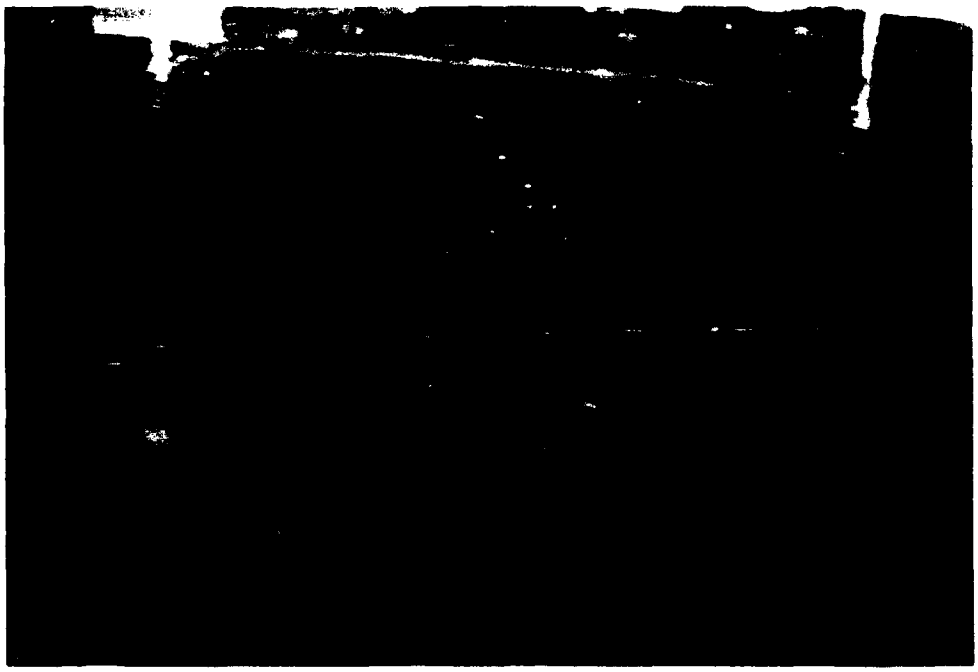

Figure 90. Treated plot of *M. spicatum* at termination of 1986 experiment with virtual absence of plant growth

Figure 91. Control plot of *M. spicatum* at termination of 1986 experiment with typical profusion of plant growth

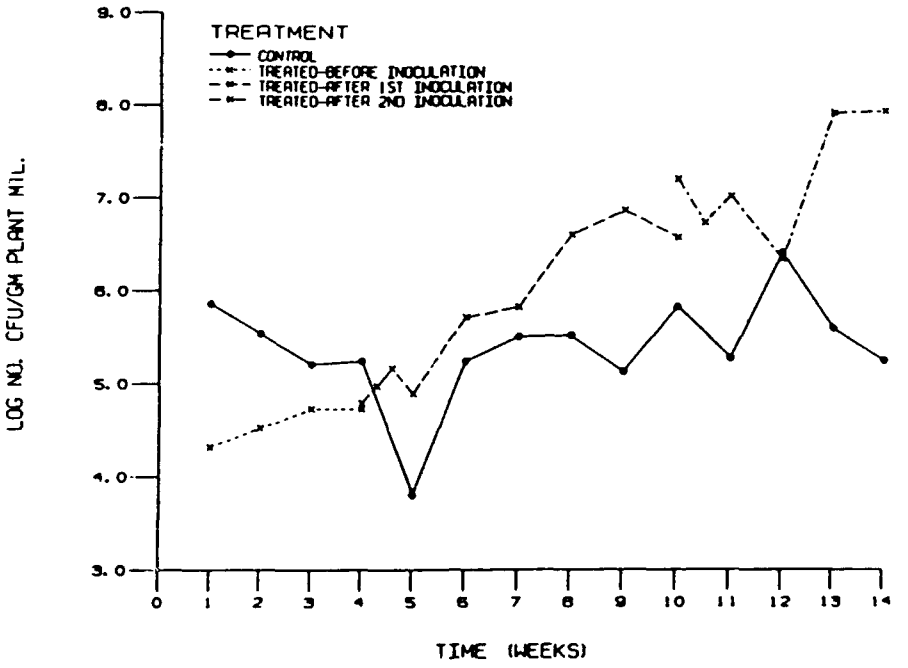

Figure 92. Bacterial populations recovered on TSA medium from control (untreated) and treated *M. spicatum* tissues in 1986 field experiment

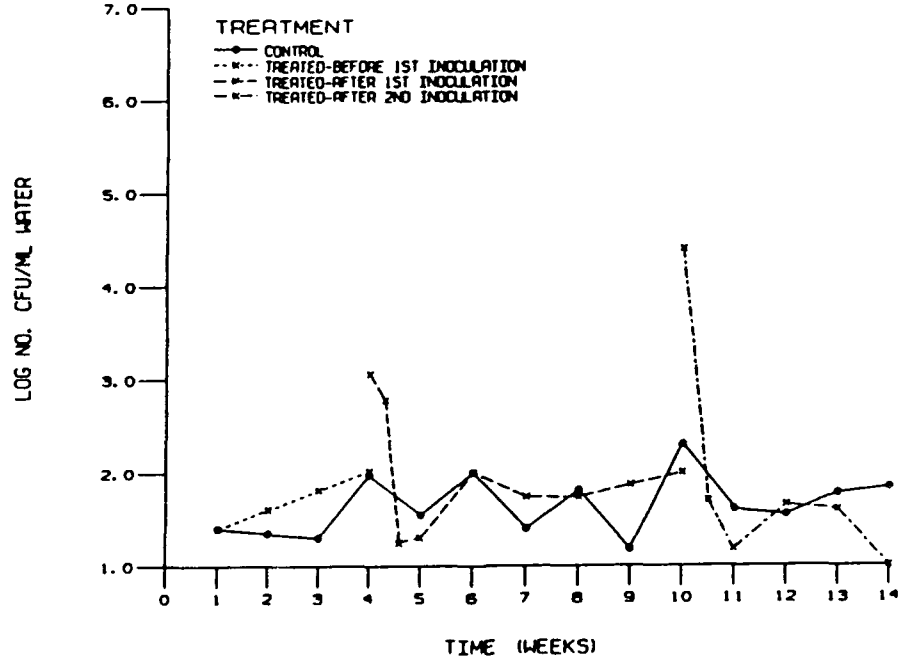

Figure 93. Bacterial populations recovered on TSA medium from control (untreated) and treated water profiles in plots containing *M. spicatum* in 1986 field experiment

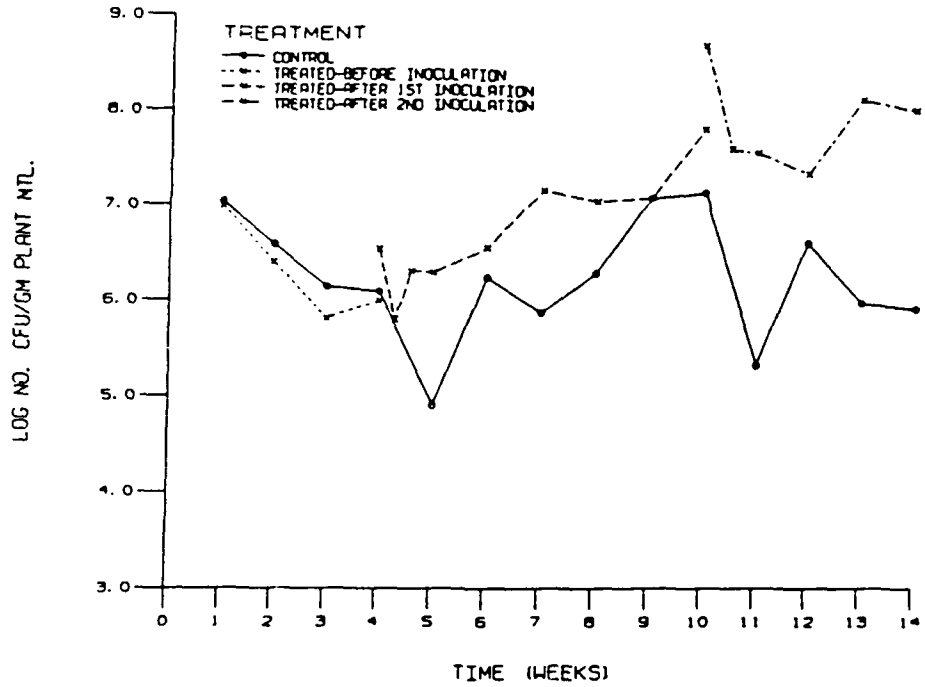

Figure 94. Populations of pectinolytic bacteria defined by recovery on PA medium from control (untreated) and treated *M. spicatum* tissues in 1986 field experiment

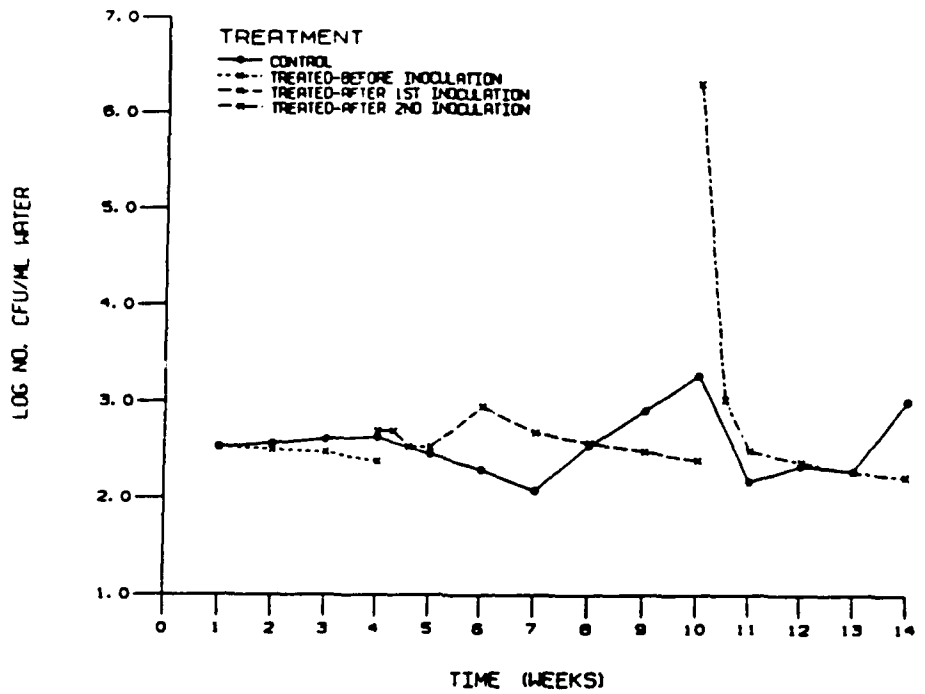

Figure 95. Populations of pectinolytic bacteria defined by recovery on PA medium from control (untreated) and treated water profiles in plots containing *M. spicatum* in 1986 field experiment

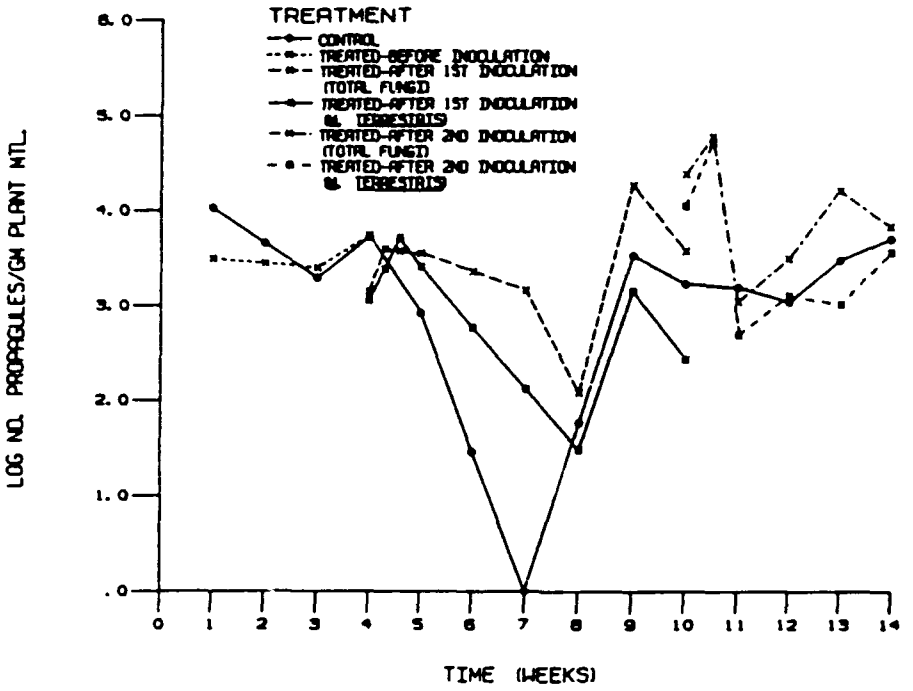

Figure 96. Fungal populations defined by recovery on MA medium from control (untreated) and treated *M. spicatum* tissues in 1986 field experiment

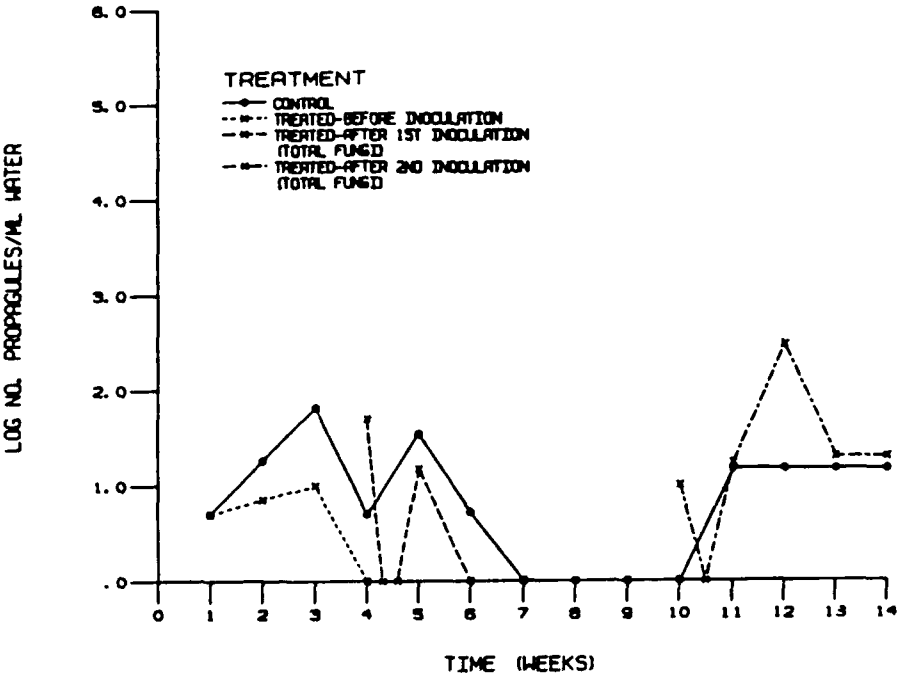

Figure 97. Fungal populations defined by recovery on MA medium from control (untreated) and treated water profiles in plots containing *M. spicatum* in 1986 field experiment

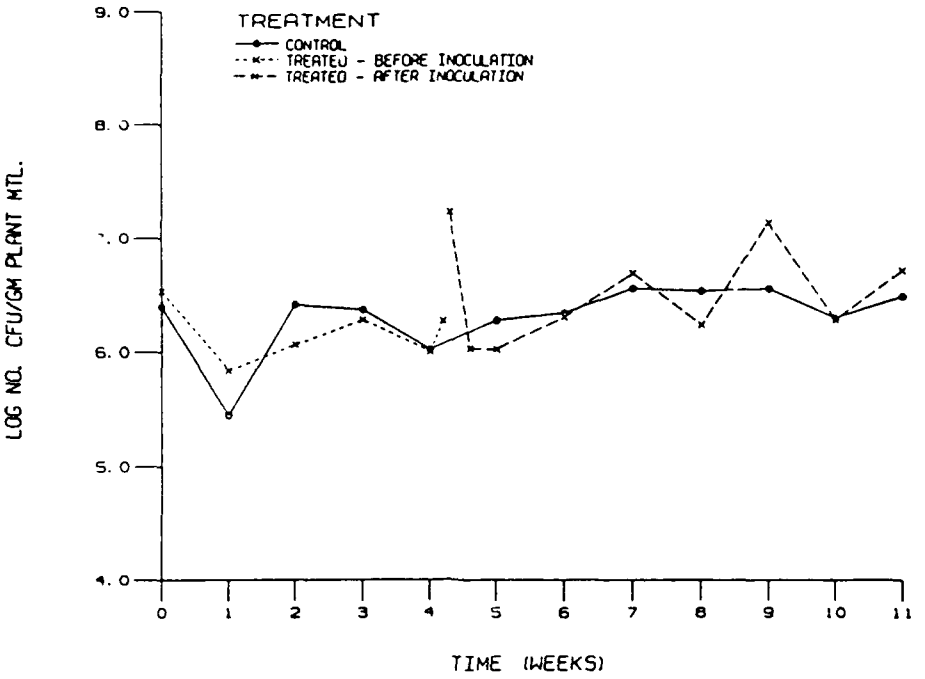

Figure 98. Microbial populations recovered on TSA medium from control and treated *M. spicatum* tips in 1987 field experiment

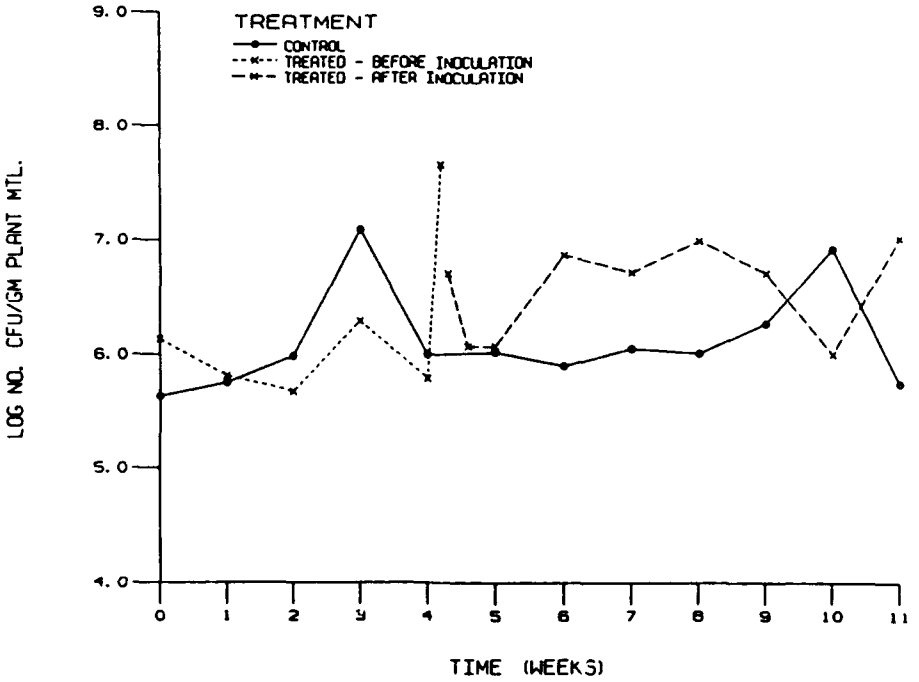

Figure 99. Microbial populations recovered on TSA medium from control and treated *M. spicatum* mid-sections in 1987 field experiment

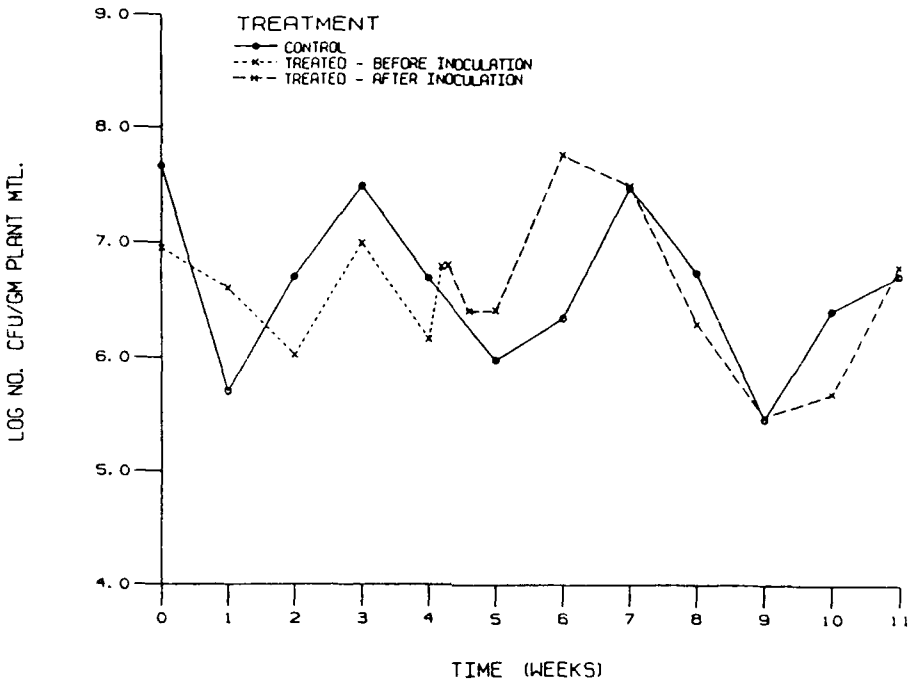

Figure 100. Microbial populations recovered on TSA medium from control and treated *M. spicatum* roots in 1987 field experiment

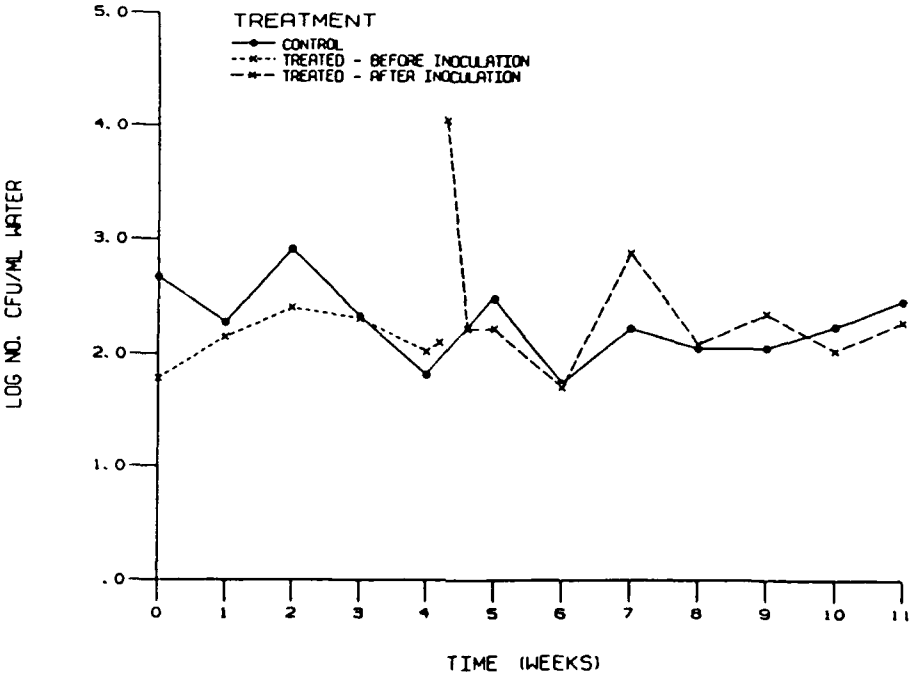

Figure 101. Microbial populations recovered on TSA medium from water profiles of control and treated plots of *M. spicatum* in 1987 field experiment

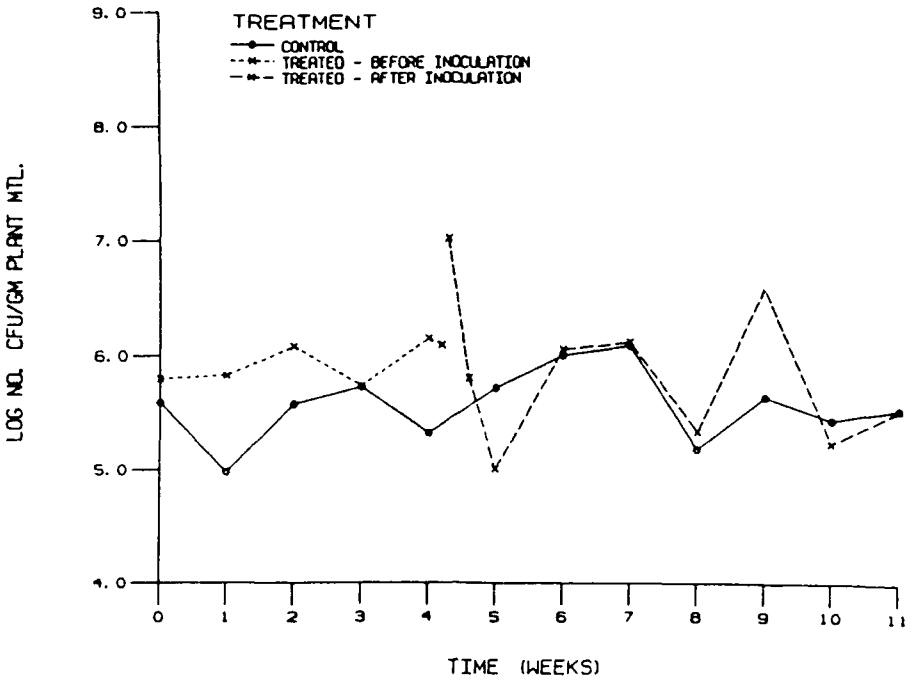

Figure 102. Populations of strongly pectinolytic bacteria recovered on PA medium from control and treated *M. spicatum* tips in 1987 field experiment

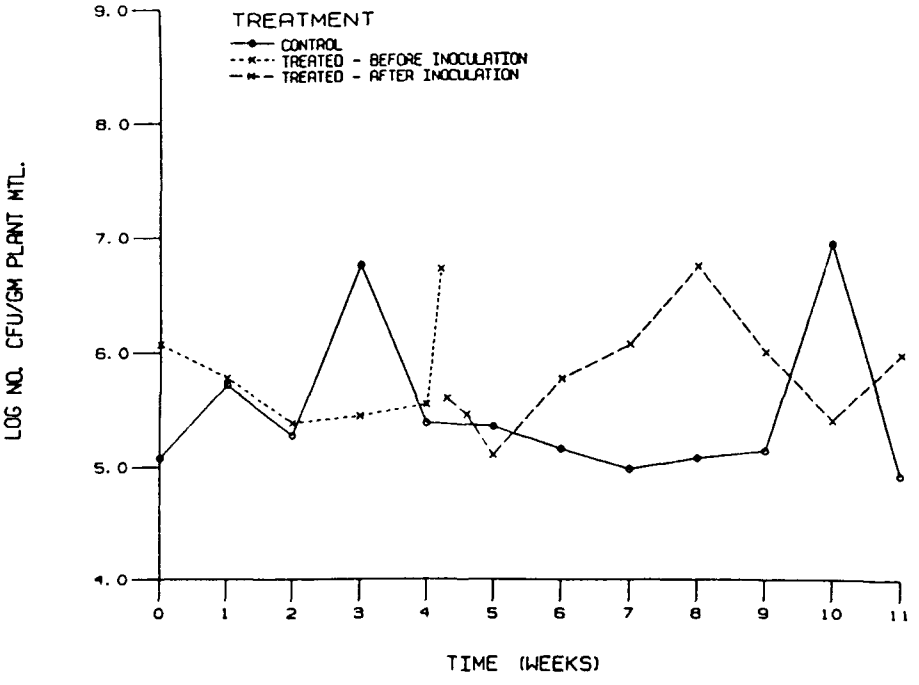

Figure 103. Populations of strongly pectinolytic bacteria recovered on PA medium from control and treated *M. spicatum* midsections in 1987 field experiment

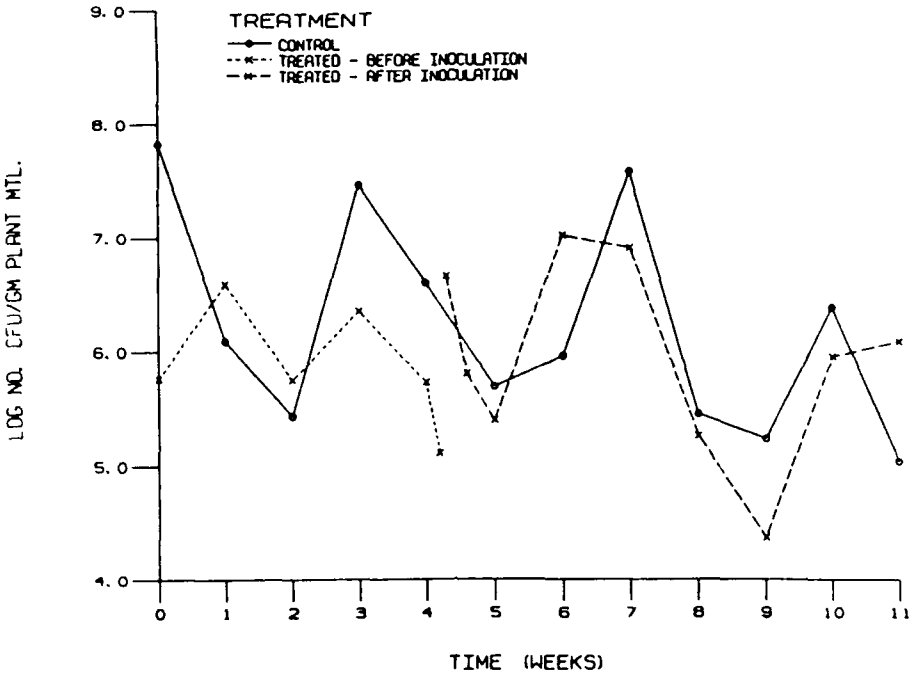

Figure 104. Populations of strongly pectinolytic bacteria recovered on PA medium from control and treated *M. spicatum* roots in 1987 field experiment

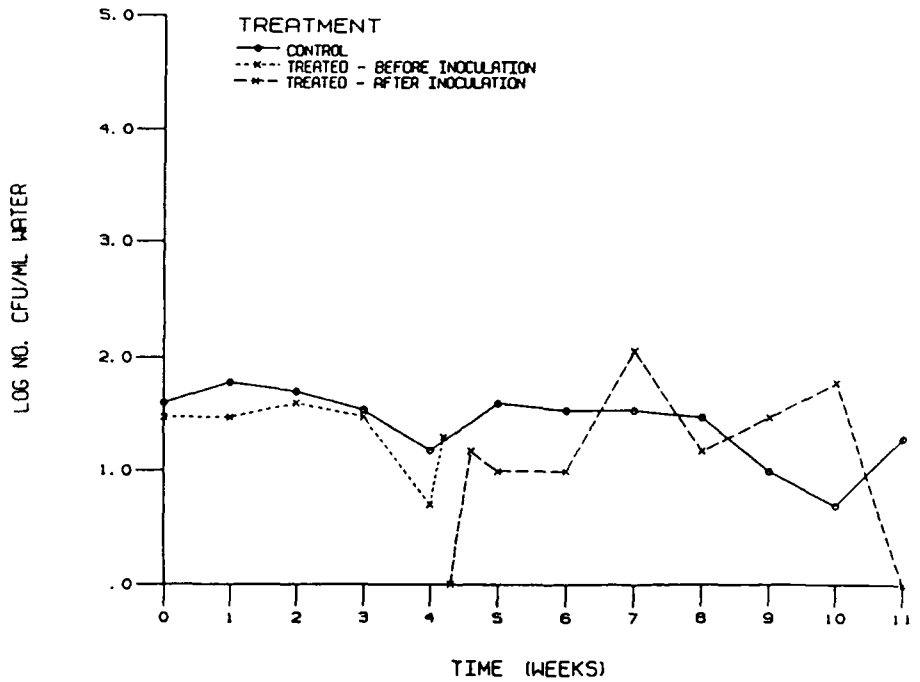

Figure 105. Populations of strongly pectinolytic bacteria recovered on PA medium from water profiles of control and treated *M. spicatum* in 1987 field experiment

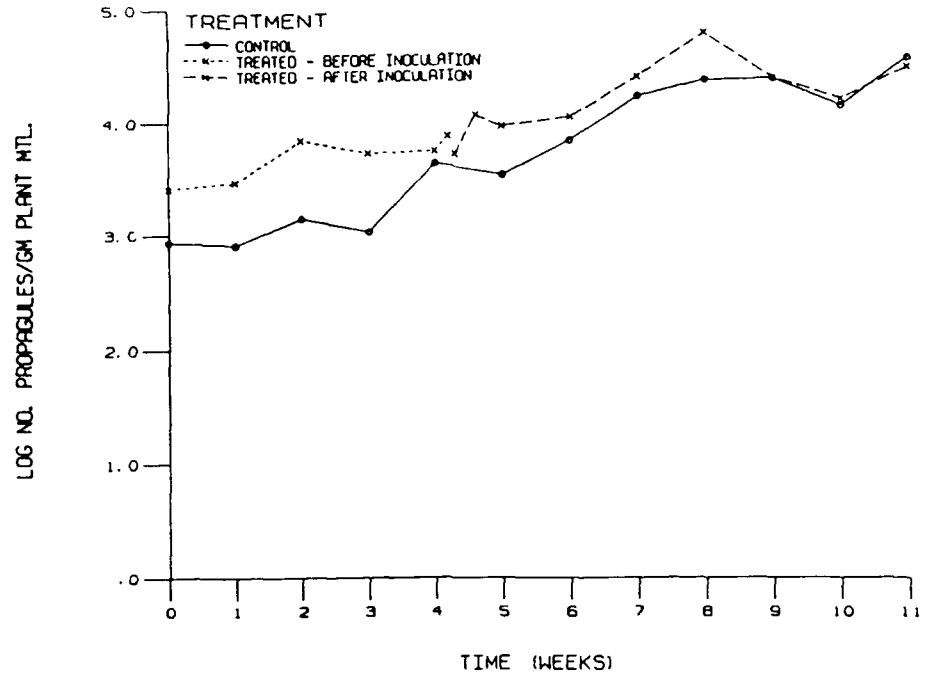

Figure 106. Total fungal populations recovered on MA medium from control and treated *M. spicatum* tips in 1987 field experiment

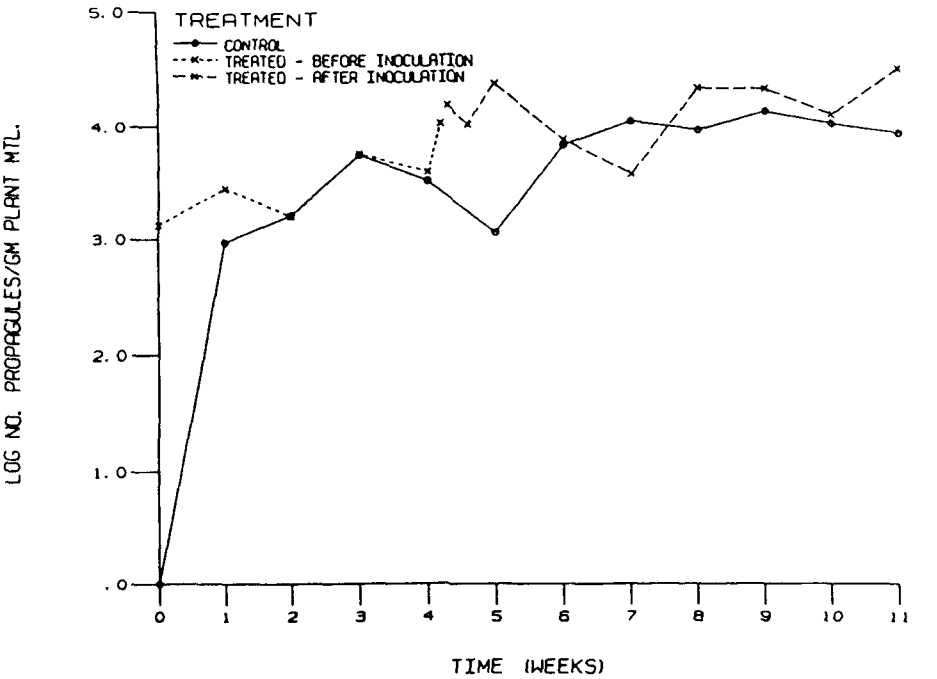

Figure 107. Total fungal populations recovered on MA medium from control and treated *M. spicatum* midsections in 1987 field experiment

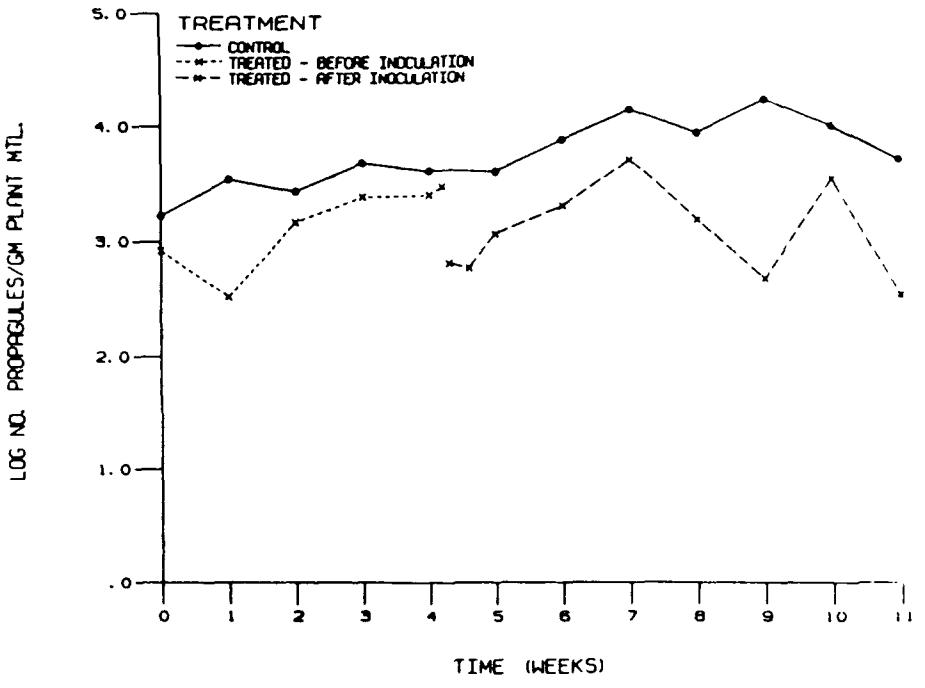

Figure 108. Total fungal populations recovered on MA medium from control and treated *M. spicatum* roots in 1987 field experiment

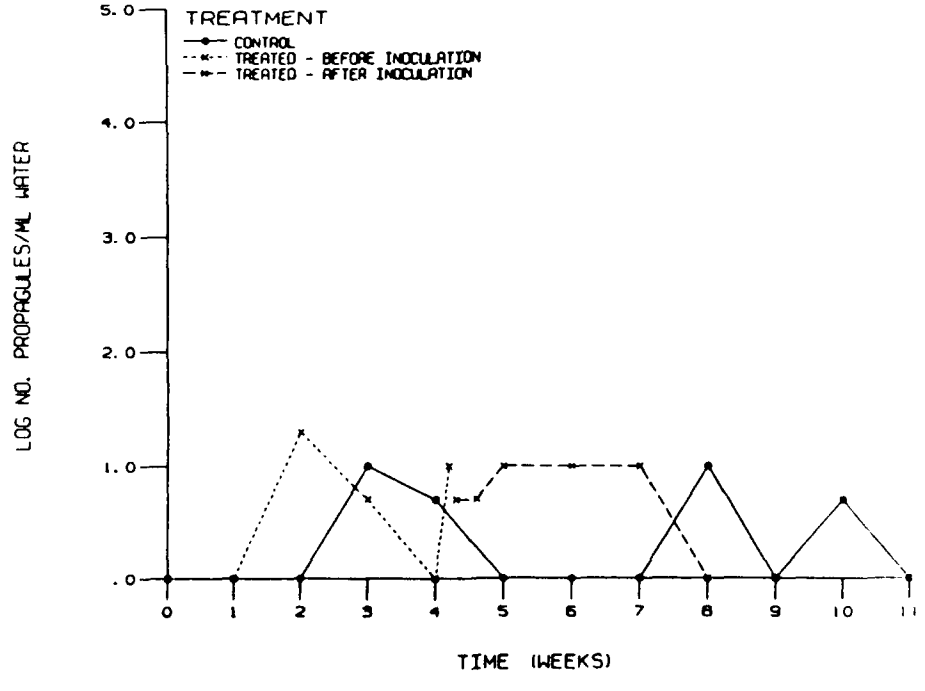

Figure 109. Total fungal populations recovered on MA medium from water profiles of control and treated plots of *M. spicatum* in 1987 field experiment

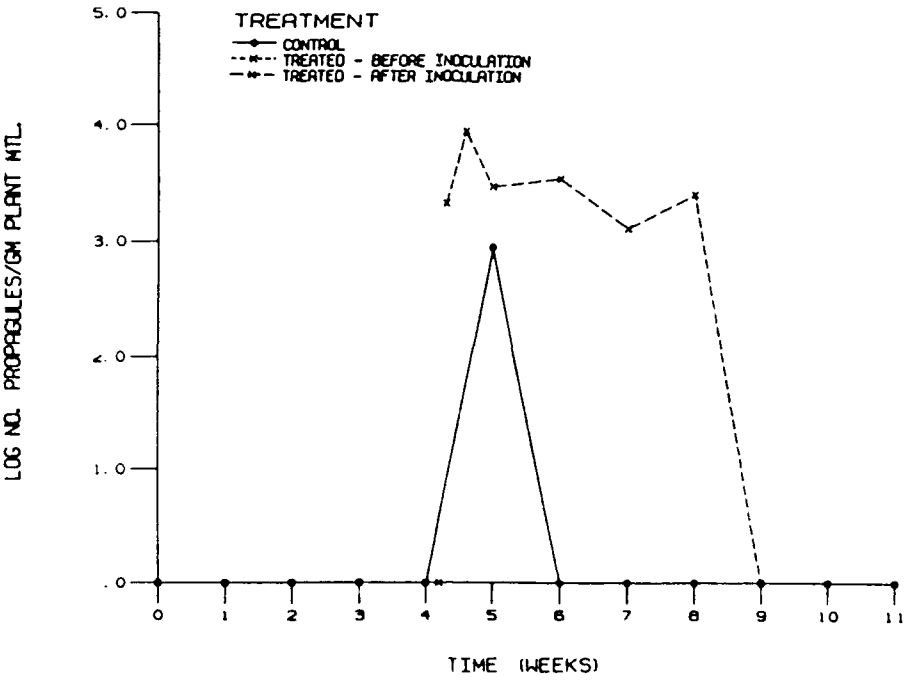

Figure 110. Populations of *M. terrestris* recovered on MA medium from control and treated *M. spicatum* tips in 1987 field experiment

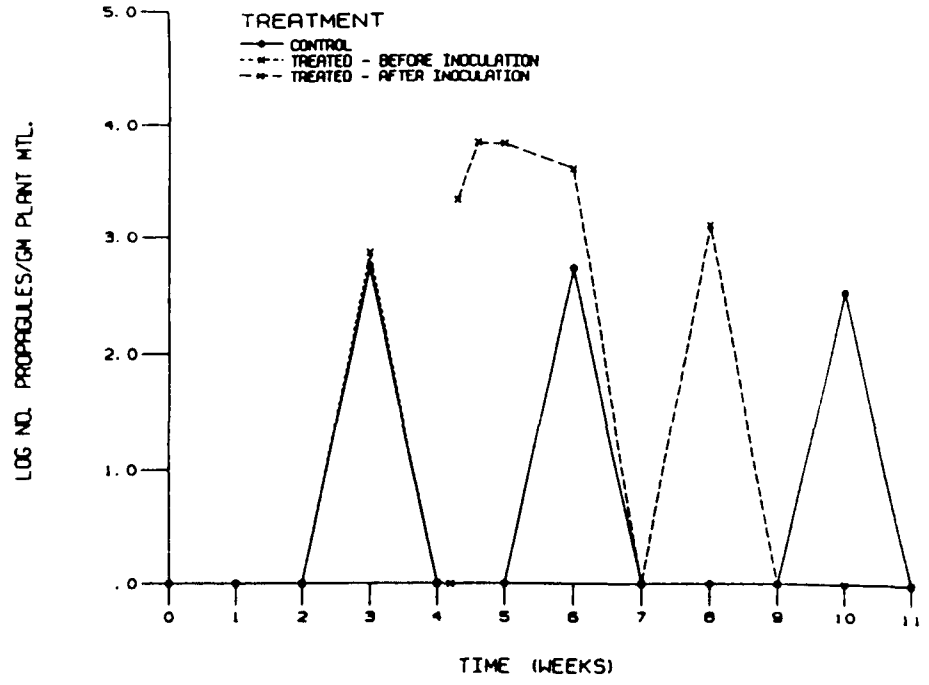

Figure 111. Populations of *M. t.* recovered on MA medium from control and treated *M. spicatum* midsections in 1987 field experiment

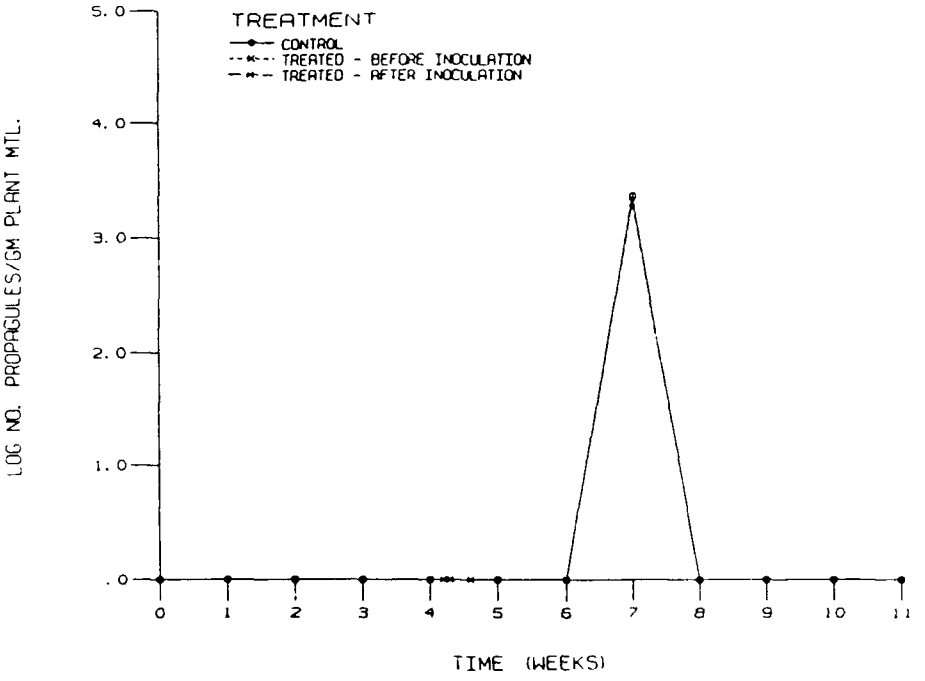

Figure 112. Populations of *M. t.* recovered on MA medium from control and treated *M. spicatum* roots in 1987 field experiment

APPENDIX A: MEDIA FORMULATIONS

Ingredients	Amount/Litre of Medium
Cellulose medium	
Phosphate buffer (1 M, pH 6.8)	20.00 ml
Yeast extract	0.20 g
$(NH_4)_2SO_4$	1.00 g
Pebble-milled cellulose*	200.00 ml
Agar (for cellulose agar)	15.00 g
Mineral salts cofactors	
Nitrilotriacetic acid	0.20 g
$MgSO_4 \cdot 7H_2O$	0.59 g
$CaCl_2 \cdot 2H_2O$	66.80 mg
$Na_2MoO_4 \cdot 2H_2O$	2.50 mg
$FeSO_4 \cdot 7H_2O$	1.98 mg
EDTA	2.50 mg
$ZnSO_4 \cdot 7H_2O$	5.00 mg
$MnSO_4 \cdot H_2O$	1.54 mg
$CuSO_4 \cdot 5H_2O$	0.39 mg
$CoCl_2 \cdot 6H_2O$	0.20 mg
$Na_2BO_4O_7 \cdot 10H_2O$	0.18 mg
Martin's medium	
KH_2PO_4	0.50 g
K_2HPO_4	0.50 g
$MgSO_4 \cdot 7H_2O$	0.50 g
Peptone	5.00 g
Dextrose	10.00 g
Rose Bengal	33.00 mg
Agar	20.0 g
Streptomycin	0.03 g
Pectin agar	
1.0 M NaOH	9.00 ml
10% $CaCl_2 \cdot 2H_2O$	6.00 ml

* 5% Whatman filter paper No. 1.

Ingredients	Amount/Litre of Medium
Pectin agar (Continued)	
Yeast extract	5.00 g
Casamino acid	1.00 g
Agar	3.00 g
Sodium polypectate	20.00 g
Polygalacturonic (agar) medium	
Phosphate buffer (0.8 M, pH 7.2)	50.00 ml
1 M $MgSO_4$	1.00 ml
0.1 M $CaCl_2$	1.00 ml
0.01 M $FeSO_4$	0.50 ml
20% $(NH_4)_2SO_4$	5.00 ml
Sodium polygalacturonate	5.00 g
Agar	15.00 g
Vitamin solution	
Thiamine•HCl	0.50 mg
Pyridoxin•HCl	0.10 mg
Folic acid	0.01 mg
Calcium pantothenate	0.50 mg
Biotin	0.005 mg
Riboflavin	0.10 mg
Nicotinic acid	0.50 mg
Vitamin B12	0.10 mg
Potato dextrose salts medium	
Potato starch	10.00 g
Dextrose	10.00 g
Yeast extract	2.00 g
Mineral salt cofactors	
$MgSO_4 \cdot 7H_2O$	200.00 mg
$CaCl_2 \cdot 2H_2O$	5.00 mg
$CoCl_2 \cdot 6H_2O$	6.00 mg
$ZnSO_4 \cdot 2H_2O$	5.00 mg
Na_2MoO_4	1.50 mg
$FeCl_2 \cdot 6H_2O$	1.70 mg
$CuSO_4 \cdot 5H_2O$	0.40 mg

Ingredients	Amount/Litre of Medium
Potato dextrose salts medium (Continued)	
$MnCl_2 \cdot 4H_2O$	3.70 mg
H_3BO_3	5.60 mg
Sporulation Medium	
Phosphate buffer (0.1 M)	100.00 ml
Phytone	2.00 g
Glucose	2.00 g
$(NH_4)_2SO_4$	3.00 g
$MgSO_4$ (anhydrous)	0.03 g
$MnSO_4 \cdot 7H_2O$	0.05 g
$ZnSO_4 \cdot 7H_2O$	7.50 mg
$CuSO_4 \cdot 5H_2O$	7.50 mg
$FeSO_4 \cdot 7H_2O$	7.50 mg
$CaCl_2 \cdot 2H_2O$	18.00 mg